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TODAY

THE

BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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FORTY-FOURTH YEAR

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11.30 A.M., daylight saving time, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the

President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeritus shall have all rights of the Trustees except that Trustees Emeritus shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen: Herewith is submitted my report as Treasurer of the Marine Biological Laboratory for the year 1931.

The accounts have been audited by Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1931, the book value of the General Endowment Fund in the hands of the Central Hanover Bank and Trust Company (of New York) as Trustee was $908,895 in securities and $53.77 in cash.

The book value of the Library Fund was $198,605 in securities, and $340 in cash.

The Reserve Fund consisted of securities of the book value of $20,868.75 and cash of $909.75.

The Retirement Fund consisted of securities of the book value of $18,896.07, invested in mortgages.

There has been little change in the other minor funds.

At this date (March 28, 1932) there is no default in any of the securities held in the above-mentioned funds.

The land, buildings, equipment, and library, excluding the Devil's Lane and Gansett properties, represented an investment of $1,642,665.24, less depreciation of $286,404.20, or a net amount of $1,356,261.04.

Current income exceeded expenses including depreciation by $2,304.35.

Over $21,000 was expended from current funds on buildings, equipment, and on books, the greater part having been expended for books.

At the end of the year, the Laboratory owed $2,349.65 in accounts payable, and $27,000 on bond and mortgage, and had over $30,000 in its bank accounts.

Following is the Balance Sheet as of December 31, 1931, and the condensed statement of income and outgo for the year, also the surplus account.

BIOLOGICAL WOODS HOLE LIBRARY

EXHIBIT A

Marine Biological Laboratory Balance Sheet, December 31, 1931

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank & Trust Company (of New York), Trustee—Schedules I-a and I-b		$1,107,893.77	
Securities and Cash—Minor Funds—Schedule II		10,411.70	$1,118,305.47
Plant Assets:			
Land—Schedule IV	$ 97,103.05		
Buildings—Schedule IV	1,207,554.14		
Equipment—Schedule IV	162,965.48		
Library—Schedule IV	175,042.57	$1,642,665.24	
Less Reserve for Depreciation		286,404.20	
		$1,356,261.04	
Securities and Cash in Reserve Fund		21,778.50	
Cash in Dormitory Building Fund		818.96	$1,378,858.50
Current Assets:			
Cash		$ 30,872.07	
Accounts—Receivable		17,998.23	
Inventories:			
Supply Department	$ 36,327.34		
Biological Bulletin	9,077.28	45,404.62	
Investments:			
Devil's Lane Property	$ 39,301.81		
Gansett Property	1.00		
Stock in General Biological Supply House, Inc.	12,700.00		
Retirement Fund Assets	18,896.07	70,898.88	
Prepaid Insurance		3,902.12	
Items in Suspense (Net)		292.12	$ 169,368.04
			$2,666,532.01

Liabilities

Endowment Funds:		
General Endowment Funds—Schedule III	$1,107,893.77	
Minor Funds—Schedule III	10,411.70	$1,118,305.47
Plant Funds:		
Donations and Gifts—Schedule III	$1,029,372.61	
Other Investments in Plant from Gifts and Current Funds	347,485.89	
	$1,376,858.50	
Mortgage, Danchakoff Estate	2,000.00	$1,378,858.50

Current Liabilities and Surplus:			
Mortgage, Devil's Lane Property		$ 25,000.00	
Accounts—Payable		2,349.65	
Woods Hole Oceanographic Institution:			
Amount received for Purchase of Books for their Library	$ 5,000.00		
Less Expenditures	4,327.63	672.37	
		$ 28,022.02	
Current Surplus—Exhibit C		141,346.02	$ 169,368.04
			$2,666,532.01

EXHIBIT B

Marine Biological Laboratory Income and Expense, Year Ended December 31, 1931

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 47,293.49		$ 47,293.49
Library Fund		10,434.77		10,434.77
Gifts		445.29		445.29
Instruction	8,110.79	9,425.00		1,314.21
Research	4,063.36	18,085.00		14,021.64
Evening Lectures	167.58		167.58	
Biological Bulletin and Membership Dues	9,060.65	9,976.43		915.78
Supply Department—Schedule V	44,834.46	57,979.24		13,144.78
Mess—Schedule VI	30,664.25	34,360.65		3,696.40
Dormitories—Schedule VII	31,739.30	13,500.86	18,238.44	
(Interest and Depreciation charged to above 3 Departments. See Schedules V, VI, and VII)	*35,453.91*			35,453.91
Dividends, General Biological Supply House, Inc.		2,032.00		2,032.00
Rent, Danchakoff Cottages	505.17	1,039.00		533.83
Rent, Microscopes		469.00		469.00
Rent, Garage, Railway, etc.		427.60		427.60
Rent, Newman Cottage	92.94	150.00		57.06
Rent, Janitor's House	118.83	390.00		271.17
Sales of Duplicate Library Sets		238.70		238.70
Interest on Bank Balances		213.76		213.76
Sundry Items		71.82		71.82
Maintenance of Plant:				
New Laboratory Expense	18,057.74		18,057.74	
Chemical and Special Apparatus	9,869.52		9,869.52	
Maintenance, Buildings and Grounds	9,147.32		9,147.32	

Library Department Expenses	8,982.70		8,982.70	
Carpenter Department Expenses	1,602.02		1,602.02	
Truck Expenses	942.02		942.02	
Sundry Expenses	282.91		282.91	
Workmen's Compensation Insurance	554.02		554.02	
General Expenses:				
Administration Expenses	15,907.89		15,907.89	
Endowment Fund Trustee	968.50		968.50	
Interest on Loans	100.00		100.00	
Bad Debts	631.24		631.24	
Naples Zoological Station	250.00		250.00	
Mosquito Fund Contribution	100.00		100.00	
Reserve for Depreciation	39,778.56		39,778.56	
Museum Expenses	3,150.40		3,150.40	
Excess of Income over Expenses carried to Current Surplus—Exhibit C	2,304.35		2,304.35	
	$206,532.61	$206,532.61	$131,035.21	$131,035.21

EXHIBIT C

Marine Biological Laboratory, Current Surplus Account
Year Ended December 31, 1931

Balance, January 1, 1931		$119,401.09
Add:		
Reserve for Depreciation charged to Plant Funds		39,778.56
Excess of Income over Expenses for Year as shown in Exhibit B		2,304.35
Excess of Gansett Property Receipts over Cost of Property and Development Expenses, etc.		1,885.95
Income of Retirement Fund	$ 734.47	
Less Pensions Paid	720.00	14.47
		$163,384.42
Deduct:		
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,		
Buildings	$ 200.11	
Equipment	3,919.20	
Library Books, etc.	17,635.22	
	$21,754.53	
Income and Reserve Fund for 1931 credited to Current Surplus and now transferred to Plant Funds	283.87	22,038.40
Balance, December 31, 1931—Exhibit A		$141,346.02

Respectfully submitted,

LAWRASON RIGGS, JR.,

Treasurer.

V. THE REPORT OF THE LIBRARIAN

The expenditures of the Library remain the same as last year, $24,000 for the Marine Biological Laboratory, and $2,123.21 for the Oceanographic Institution. The $5,000 appropriated by the last-named in March, 1930, will be expended before the end of their fiscal year of February 29, 1932, for necessary books and back sets. A separate rendering of this account is kept on file. The only item in the regular $24,000 necessary to mention is that for current serials, which has, during 1930 and 1931, overreached the $5,000 assigned in 1929, by $72.94 and $631.07 respectively. In 1932, $6,000 is allowed for current serials. This increase was, however, anticipated in 1929 and the definite plan made to enlarge the subscriptions each year by a sum taken from the $8,200 assigned in 1929 for back sets. Until the back sets of 450 different serials are purchased which are very necessary to the Library before it is on a par with the finest libraries of the country, it is the judgment of the Librarian that the current expenditure on serials should not greatly exceed $6,000, thus leaving for a number of years the $7,000 that will, if wisely spent in completing back sets, increase the usefulness and monetary value of the Library much beyond the intrinsic sum spent. The full list of over 1,000 serial sets imperfect in the back holdings or in current receipts has been carefully scrutinized during the winter, 1931–32, and definite decision has been made, based on various different and combined reasons, to borrow or to leave unfilled about half of the number. Since every serial title in the Library, regardless of the method of acquisition and of its value, has been automatically recorded when deficient, the weeding out of half of these seems reasonable when choice was made without regard to any definite number to be retained or discarded, but the decisions are subject, of course, to future revision on the part of the investigators. Before the date of the summer report an estimate will have been made of the definite sum of money that will be necessary in order that the Library shall record in its catalogue 1,319 complete serial publications as against 869 perfectly complete now. A sum of $1,000 should always be available under this item, however, since new current serials will come on our list entailing the purchase in most cases of previously issued volumes.

A statement of the holdings of the Library shows the following: 1,080 currently received serials; 33,780 volumes; and 69,851 reprints. A detail of the current serials, 391 subscriptions, 481 exchanges, 208 gifts, shows an increase over last year in current paid subscriptions by 45, of which 14 were for the Woods Hole Oceanographic Institution, and 39 new exchanges. The low total increase from the year 1930 to

1931 from 1,060 to 1,080 is due to a rigid elimination of gifts and exchanges not regularly received, and will be enhanced another year when a selection will have been made of those important to us. As a temporary measure, a great number of incomplete and irregularly received United States Government publications have been entirely segregated from the serials, pending necessary decision as to their value for us.

The number of bound volumes added to the Library in 1931 was 1,923; of these 1,576 were serials, 48 sets having been filled in, seven of these for the Oceanographic, 33 partially filled in, two of these for the Oceanographic, and 347 were books. The unusual number of 156 books was purchased by us, using, in addition to the budgeted $300, a sum of $500 from the "back sets" money, and 95 were purchased for the Oceanographic Institution. In calling attention to the 33,780 volumes in the Library at the present time, it will not be out of place to record the fact that the accessioned number of volumes is a misleading figure as to the actual number of serial volumes the Library contains. For the sake of economy in binding and in order to coördinate thickness and height in volumes and make them look well as they stand on the shelves, we have during the past ten years increased rather than diminished the number in which we bind two or more volumes in one, thus reducing consistently the accessioned number below the actual. The shelf space occupied by the volumes indicates, indeed, a figure much more nearly 40,000 than the correct 33,780. The estimated capacity of the shelving space in the Library as given by the architect at 100,000 is correct for the small size volumes of our serial sets. Since one of the five floors of stacks must be reserved for reprints, the inference may be made that the space for serials and books will be completely exhausted only when the number 33,780 volumes, bound and accessioned in accordance with our present method, is doubled.

The reprints added to the files this year were 5,620; 2,745 of these were catalogued from the gift of Professor Metcalf and the Sidney I. Smith collection, and 2,875 total the year's current receipts. The majority of current reprints are from the authors, but the "Collected Papers" of 63 different laboratories are regularly received. Of these sets of reprints, 27 are complete and 36 are incomplete in the back files If these were counted as serial publications our list of current serial receipts would be 1,143 instead of 1,080. Of the reprints 1,828 are bound. Three hundred and seventy-five of these volumes contain 10–40 separates.

While the gifts of books from publishers are many fewer than some years ago when special effort was made to secure presentation copies direct from the publisher, the number from authors was exceptionally high. This was due to Professor Baitsell and to Mr. Ware Cattell, who very generously presented to the Library the books reviewed during the summer in the "Collecting Net." A book plate records the gift as a joint one from the author, publisher, reviewer, and the "Collecting Net." The books presented to the Library this year are briefly enumerated below. These gifts are acknowledged with pleasure, and with very warm thanks.

"Collecting Net"	19
Authors	14
P. Blakiston's Son and Co.	4
Bruce Publishing Co.	1
Chicago University Press	1
Harvard University Press	1
John Wiley & Sons, Inc.	1
Alfred A. Knopf	1
Lea & Febiger	1
Macmillan Co.	2
W. B. Saunders & Co.	1
Dr. Henry McE. Knower	3
Dr. C. A. Cheever	1
National Academy of Sciences	1
Eastman Kodak Company	1

MARINE BIOLOGICAL
WOODS HO
LIBRAR
MASS.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I beg to submit herewith a report of the forty-fourth session of the Marine Biological Laboratory for the year 1931.

1. *Attendance.* The attendance for 1931 was unexpectedly large, the total number of investigators for the season being 362 as compared with 337 in 1930, the next-highest year. The tabular view of attendance on page 29 shows in detail the relation of the past season to the four immediately preceding it. Particularly noteworthy is the large number of institutions represented in 1931, this being 137 as compared with 126 in 1930 and 123 in 1929.

Of considerable importance to the Laboratory from a practical standpoint is the peak attendance. It is this, rather than the total attendance, which determines the degree of crowding of the Laboratory, the Mess, and the available living accommodations. Following the high peak of 286 in 1928, a measure of relief was afforded for a time by a new arrangement of the courses, which tended to spread the attendance over a longer period; and, for several years, a further growth of the total attendance occurred without evidences of undue crowding. During the past season, however, the peak attendance suddenly increased by 43 over that for 1930 and surpassed by 20 even the previous high record of 1928. Since it seems impracticable at present for the Laboratory to increase the available research and living accommodations, the attention of investigators is again invited to the advantages of utilizing to a greater extent than at present the early and the late parts of the season. The following tabulation of attendance on selected dates for the past five years gives a clear view of the general situation.

		1927	1928	1929	1930	1931
May	30	7	15	9	6	6
June	10	50	64	55	50	51
“	20	114	140	139	153	153
“	30	212	240	197	208	217
July	10	247	281	238	253	258
“	20	247	282	242	250	273
“	30	245	272	249	253	281
August	10	234	250	256	254	302
“	20	208	226	243	245	280
“	30	168	183	220	204	239
September	10	110	112	157	122	136
“	20	50	43	59	44	69
“	30	12	14	14	8	14

2. *The Report of the Treasurer.* This report shows a slight increase in the total assets of the Laboratory over the preceding year, the figures being $2,666,532.01 for 1931 as compared with $2,660,559.11 for 1930. Though the income for 1931 was less than that for 1930 ($206,532.61 as compared with $210,110.86), a reduction of expenses from $213,878.11 to $204,228.36 permitted for the first time in six years the appearance of an excess of income over expenses, including depreciation charges. While the Laboratory was not greatly affected during 1931 by the existing financial depression, the indications at the time of the preparation of this report are that the year 1932 will show a serious decrease in the subscriptions received from coöperating and subscribing institutions and somewhat smaller decreases in the income from the Supply Department and from the permanent endowment fund. It is hoped, however, that by means of all practicable economies it may be possible to preserve the very gratifying condition of financial soundness that has characterized the Laboratory for so many years, even though the exceptionally favorable showing for 1931 may not again be equalled for some time.

3. *The Report of the Librarian.* The growth of the Library in 1931 has continued at approximately the same rate as that for the past six years. Its development since 1925 may perhaps best be shown by means of the following figures taken in part from the reports of previous years.

Particularly noteworthy is the fact that 869 sets of serial publications are now complete; these include most of the sets in common use.

	1925	1926	1927	1928	1929	1930	1931
Serials received currently	500	628	764	874	985	1060	1080
Total number of bound volumes	15000	18200	22800	26500	28300	31500	33800
Reprints	25000	38000	43000	51000	59000	64000	70000

4. *Publications.* During the early years of the Laboratory, a record was kept of all the published scientific work that issued from it. With its continued growth, however, the difficulty of keeping such a record became very great and the practice was discontinued about twenty years ago. The development in recent years of a trained library staff accustomed to the collection and the cataloguing of reprints has again made it possible, not merely to keep a record of all publications, but to bind and index the papers themselves in such a way that they may be readily available both for the use of investigators and of other persons interested in the scientific accomplishments of the Laboratory. Through the kind coöperation of the investigators concerned, approximately 100 papers, based on work done at the Laboratory and published in 1930, are now ready for binding. It is planned to prepare similar sets for each subsequent year; and it is to be expected that the value of these sets, both historical and scientific, will in time become very great. The thanks of the Laboratory are due to all who have so generously contributed the necessary reprints of their papers.

5. *Lectures and Scientific Meetings.* Twelve evening lectures were delivered during 1931, including the special Reynold A. Spaeth Memorial Lecture by Professor Ross G. Harrison. In addition, there were held 12 other meetings, at which 63 shorter scientific papers were presented and discussed.

A successful innovation in 1931 was the special scientific session held on September 3 and devoted exclusively to work completed at the Laboratory during the current season. Twenty-two papers conveniently grouped by subjects were presented at this session, which occupied the greater part of the day. The remainder of the day was devoted to the inspection of demonstrations of work in progress at the Laboratory. The titles of the various lectures and shorter papers for 1931, which give a very representative cross-section of the work of the Laboratory for that year, are listed on pages 31 to 36.

6. *Supply Department and Museum.* The period of management of the Supply Department by the General Biological Supply House

having terminated in August, 1931, Mr. James McInnis, who for the past year had acted as Resident Manager, was placed in full charge of the Department. Under his management the Supply Department has continued, in spite of generally unfavorable business conditions, to provide an important part of the revenue needed for the running of the Laboratory, besides filling with efficiency its primary function of supplying living material to investigators.

During the past year very gratifying progress has been made in the development of a working museum by its Curator, Mr. George M. Gray. A large number of representatives of the local fauna, properly preserved and labeled, are now available for the use of investigators who wish to identify material of their own or whose work in other ways requires museum facilities. In addition to the preservation of material, particular attention has been given during the year to the accumulation and tabulation of data on the geographical and seasonal distribution of the local forms. Mr. Gray's long experience with the fauna of the Woods Hole region gives him exceptional qualifications for carrying out this very important part of the work of the Museum.

7. *Meeting of the Corporation.* At the annual meeting of the Corporation, held on August 11, the report of a special committee appointed a year previously to consider changes in the method of nominating trustees was considered, and the following recommendations of the Committee were adopted:

> 1. "The Corporation affirms its position that instruction is a fundamental part of the work of the Laboratory and hence this work should be adequately represented upon the Board of Trustees."
> 2. "That the Committee of the Corporation for nomination of Trustees consist of five members, of whom not less than two shall be Trustee members and not less than two shall be non-Trustee members of the Corporation."
> 3. "That on or about July first of each year, the Clerk shall send a circular letter to each member of the Corporation giving the names of the Nominating Committee and stating that this Committee desires suggestions regarding nominations."
> 4. "That the Nominating Committee shall post the list of nominations at least one week in advance of the annual meeting of the Corporation."

A recommendation that no trustee shall be eligible for re-election until one year after the expiration of the term for which he was elected was discussed at length, but the motion to accept it was lost. The following new trustees were elected by the Corporation: L. V. Heilbrunn (Class of 1935), H. B. Goodrich (Class of 1933).

Following the request of Dr. Gary N. Calkins, who for 19 years had ably served the Laboratory as Clerk of the Corporation, that his name be not presented to the Corporation for re-election, the Nominating Committee selected in his place Dr. Charles Packard, of Columbia University, who was duly elected to the position, thereby becoming at the same time a Trustee ex-officio. Dr. Calkins, who on his retirement from office ceased to be a Trustee ex-officio, was elected to fill one of the regular positions in the Class of 1935. A resolution of appreciation of the long and valued services of Dr. Calkins as its Clerk was adopted by a rising vote of the Corporation.

8. *Changes in the By-laws.* At the annual meeting of the Board of Trustees two changes in the By-laws were approved. Those parts of the By-laws affected by the changes in question now read, as amended:

1. " The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11:30 A.M., daylight saving time, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years."

2. " Trustees ex efficio and Emeritus shall have all rights of the Trustees except that Trustees Emeritus shall not have the right to vote."

9. *Gifts.* Appreciative acknowledgment is made of the assistance of the Committee on the Effects of Radiation of the National Research Council and of various persons, who through the efforts of this Committee became interested in the needs of the Laboratory, in connection with investigations requiring the use of X-rays and other types of radiation. Without this assistance much of the work accomplished in this field during the past year would have been impossible. The thanks of the Laboratory are also due to Mrs. J. C. Hemmeter for the gift of scientific apparatus formerly belonging to her husband, the late Dr. John C. Hemmeter, and to Mr. Ware Cattell for the continuation in 1931 of the Collecting Net Scholarships and for the gift to the Library of a considerable number of books.

There are appended as parts of this report:

1. The Staff, 1931.
2. Investigators and Students, 1931.
3. A Tabular View of Attendance, 1927–1931.
4. Subscribing and Coöperating Institutions, 1931.
5. Evening Lectures, 1931.
6. Shorter Scientific Papers, 1931.
7. Members of the Corporation, August, 1931.

Respectfully submitted,

M. H. Jacobs,
Director.

1. THE STAFF, 1931

Merkel H. Jacobs, *Director,* Professor of General Physiology, University of Pennsylvania.

Associate Director: ——

ZOÖLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
E. G. Conklin, Professor of Zoölogy, Princeton University.
Caswell Grave, Professor of Zoölogy, Washington University.
H. S. Jennings, Professor of Zoölogy, Johns Hopkins University.
Frank R. Lillie, Professor of Embryology, University of Chicago.
C. E. McClung, Professor of Zoölogy, University of Pennsylvania.
S. O. Mast, Professor of Zoölogy, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoölogy, Harvard University.
E. B. Wilson, Professor of Zoölogy, Columbia University.
Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. Instruction

J. A. Dawson, Assistant Professor of Biology, College of the City of New York.
T. H. Bissonnette, Professor of Biology, Trinity College.
E. C. Cole, Associate Professor of Biology, Williams College.
O. E. Nelsen, Instructor in Zoölogy, University of Pennsylvania.
A. W. Pollister, Instructor in Zoölogy, Columbia University.
L. P. Sayles, Instructor in Biology, College of the City of New York.
A. E. Severinghaus, Assistant Professor of Anatomy, College of Physicians and Surgeons, Columbia University.

Junior Instructors

B. R. Coonfield, Professor of Biology, Southwestern College.
C. E. Hadley, Assistant Professor of Biology, New Jersey State Teachers College at Montclair.

PROTOZOÖLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
Rachel Bowling, Instructor in Zoölogy, Columbia University.
W. Byers Unger, Assistant Professor of Zoölogy, Dartmouth College.

EMBRYOLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Hubert B. Goodrich, Professor of Biology, Wesleyan University.
Benjamin H. Grave, Professor of Biology, De Pauw University.
Leigh Hoadley, Professor of Zoölogy, Harvard University.
Charles Packard, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.
Harold H. Plough, Professor of Biology, Amherst College.

PHYSIOLOGY

I. Investigation

Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.
Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.
Ralph S. Lillie, Professor of General Physiology, University of Chicago.
Albert P. Mathews, Professor of Biochemistry, University of Cincinnati.

II. Instruction

Teaching Staff

William R. Amberson, Professor of Physiology, University of Tennessee.
Philip Bard, Assistant Professor of Physiology, Harvard Medical School.
Ralph W. Gerard, Assistant Professor of Physiology, University of Chicago.
Laurence Irving, Associate Professor of Physiology, University of Toronto.
Leonor Michaelis, Member of the Rockefeller Institute, New York City.
Margaret Sumwalt, Assistant Professor of Physiology, Woman's Medical College of Pennsylvania.

Special Lecturers

Edwin J. Cohn, Associate Professor of Physical Chemistry, Harvard University.
Henry J. Fry, Associate Professor of Biology, Washington Square College, New York University.
E. Newton Harvey, Professor of Physiology, Princeton University.
Selig Hecht, Professor of Biophysics, Columbia University.
Merkel H. Jacobs, Professor of General Physiology, University of Pennsylvania.
Balduin Lucké, Associate Professor of Pathology, University of Pennsylvania.

BOTANY

I. Investigation

B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.
C. E. Allen, Professor of Botany, University of Wisconsin.
S. C. Brooks, Professor of Zoölogy, University of California.
Ivey F. Lewis, Professor of Biology, University of Virginia.
Wm. J. Robbins, Professor of Botany, University of Missouri.

II. Instruction

William Randolph Taylor, Professor of Botany, University of Pennsylvania.

Hannah T. Croasdale, Biological Abstracts, University of Pennsylvania.

James P. Poole, Professor of Evolution, Dartmouth College.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Librarian.

Deborah Lawrence, Secretary.

Hester Ann Bradbury, Hazel Blanchard, Mary A. Rohan, Assistants.

CHEMICAL SUPPLIES

Oscar W. Richards, Instructor in Biology, Yale University.

SCIENTIFIC APPARATUS AND TECHNICAL SUPPLIES

Samuel E. Pond, Assistant Professor of Physiology, Schools of Medicine and Dentistry, University of Pennsylvania, in charge.

A. R. Apgar, Photographer.
J. D. Graham, Glassblower.
Lester F. Boss, Mechanician.
P. H. Liljestrand, Assistant.

MUSEUM

George M. Gray, Curator.

SUPPLY DEPARTMENT

James McInnis, Manager.
A. M. Hilton, Collector.
Milton B. Gray, Collector.
Walter Kahler, Collector.
Geoffrey Lehy, Collector.
A. W. Leathers, Shipping.

BOATS

John J. Veeder, Captain.
E. M. Lewis, Chief Engineer.

F. M. MacNaught, Business Manager.

Herbert A. Hilton, Superintendent of Buildings and Grounds.

Thomas Larkin, Superintendent of Mechanical Department.

William Hemenway, Carpenter.

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2. INVESTIGATORS AND STUDENTS, 1931

Independent Investigators

Adams, A. Elizabeth, Professor of Zoölogy, Mount Holyoke College.

Addison, William H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.

Allee, W. C., Professor of Zoölogy, University of Chicago.

Amberson, William R., Professor of Physiology, University of Tennessee.

Anderson, Rubert S., Research Associate, Princeton University.

Armstrong, Philip B., Assistant Professor of Anatomy, Cornell University Medical College.

Astrom, I. Elisabeth, Class Assistant, University of Toronto.
Austin, Mary L., Instructor in Zoölogy, Wellesley College.
Bailey, Percy L., Jr., Instructor in Physiology, College of the City of New York.
Baitsell, George A., Professor of Biology, Yale University.
Bakwin, Harry, Assistant Clinical Professor, New York University.
Bakwin, Ruth Morris, Instructor in Pediatrics, New York University.
Ball, Eric G., Instructor in Physical Chemistry, Johns Hopkins University Medical School.
Ballard, William W., Instructor in Zoölogy, Dartmouth College.
Bard, Philip, Assistant Professor of Physiology, Harvard Medical School.
Barrón, E. S. Guzman, Research Associate, University of Chicago.
Barth, L. G., National Research Council Fellow, University of Chicago.
Beams, H. W., Assistant Professor of Zoölogy, State University of Iowa.
Belkin, Morris, Instructor in Biology, Washington Square College, New York University.
Beutner, R., Professor of Pharmacology, University of Louisville, School of Medicine.
Bissonnette, Thomas H., Professor of Biology, Trinity College.
Bodansky, Oscar, Instructor in Pediatrics, New York University and Bellevue Hospital.
Borodin, D. N., 621 West 42d Street, New York City.
Bowling, Rachel, Instructor in Zoölogy, Columbia University.
Bradley, H. C., Professor of Physiological Chemistry, University of Wisconsin.
Bridges, Calvin B., Research Assistant in Genetics, Carnegie Institution of Washington.
Brinley, Floyd J., Assistant Professor of Zoölogy, North Dakota State College.
Bronfenbrenner, J., Professor of Bacteriology and Public Health, Washington University Medical School.
Budington, Robert A., Professor of Zoölogy, Oberlin College.
Calkins, Gary N., Professor of Protozoölogy, Columbia University.
Cannan, Robert K., Professor of Chemistry, New York University and Bellevue Hospital Medical College.
Carpenter, Russell L., Instructor in Anatomy, College of Physicians and Surgeons, Columbia University.
Carver, Gail L., Professor of Biology, Mercer University.
Castle, William A., Instructor in Biology, Brown University.
Cattell, Ware, New York University.
Chambers, Robert, Research Professor and Chairman of Department of Biology, Washington Square College, New York University.
Cheever, Clarence A., Member, Boston Society of Natural History.
Cheney, Ralph H., Chairman, Biology Department, Long Island University.
Chidester, F. E., Professor of Zoölogy, West Virginia University.
Christie, Jesse R., Associate Nematologist, United States Department of Agriculture.
Clark, Eleanor Linton, Research Assistant in Anatomy, University of Pennsylvania.
Clark, Eliot R., Director of Department of Anatomy, University of Pennsylvania.
Clowes, G. H. A., Director, Lilly Research Laboratories.
Cobb, N. A., Principal Nematologist, United States Department of Agriculture.
Coe, Wesley R., Professor of Biology, Yale University.
Cole, Elbert C., Associate Professor of Biology, Williams College.
Cole, Kenneth S., Assistant Professor of Physiology, College of Physicians and Surgeons, Columbia University.
Conklin, Edwin G., Professor of Biology, Princeton University.
Coonfield, B. R., Instructor in Zoölogy, Brooklyn College of the City of New York.

Copeland, Manton, Professor of Biology, Bowdoin College.
Cowdry, E. V., Professor of Cytology, Washington University.
Cowles, R. P., Professor of Zoölogy, Johns Hopkins University.
Curtis, W. C., Professor of Zoölogy, University of Missouri.
Curwen, Alice O., Instructor in Histology and Embryology, Woman's Medical College of Pennsylvania.
Danks, W. B. C., Government Officer of Kenya Colony.
Darrah, Wm. C., Fellow in Paleobotany, Carnegie Museum, University of Pittsburgh.
Dawson, Alden B., Associate Professor of Zoölogy, Harvard University.
Dawson, J. A., Assistant Professor of Biology, College of the City of New York.
Dodds, Gideon S., Professor of Histology and Embryology, West Virginia University.
Dolley, William L., Jr., Professor of Biology, University of Buffalo.
Donaldson, Henry H., Member, Wistar Institute.
DuBois, Eugene F., Professor of Medicine, Cornell University Medical College.
Dunbar, Francis F., Graduate Assistant in Zoölogy, Columbia University.
Edwards, Dayton J., Associate Professor of Physiology, Cornell University Medical College.
Einarson, Larus, Research Fellow of the Rockefeller Foundation, Harvard University Medical School.
Failla, G., Physicist, Memorial Hospital, New York City.
Favilli, Giovanni, First Assistant in the Institute of General Pathology, Royal University, Florence, Italy.
Fogg, Lloyd C., Instructor in Biology, Washington Square College, New York University.
Fraser, Doris A., Assistant in Anatomy, University of Pennsylvania.
French, Charles S., Graduate Student and Assistant in General Physiology, Harvard University.
Fry, Henry J., Professor of Biology, Washington Square College, New York University.
Furth, Jacob, Associate in Pathology, The Henry Phipps Institute.
Garrey, W. E., Professor of Physiology, Vanderbilt University School of Medicine.
Gayet, René, Directeur Adjoint Laboratoire de Physiologie Pathologique, Collège de France.
Geiman, Quentin M., Director of Science Department, Swarthmore Preparatory School.
Gelfan, Samuel, Assistant Professor of Physiology and Pharmacology, University of Alberta.
Gerard, R. W., Associate Professor of Physiology, University of Chicago.
Gilson, Lewis E., Instructor in Biochemistry, University of Cincinnati.
Goldforb, A. J., Professor of Biology, College of the City of New York.
Goodrich, Hubert B., Professor of Biology, Wesleyan University.
Grave, B. H., Professor of Zoölogy, DePauw University.
Grave, Caswell, Professor of Zoölogy, Washington University.
Green, Arda A., Research Fellow in Physical Chemistry, Harvard University Medical School.
Greenwood, Alan W., Lecturer, Institute of Genetics, University of Edinburgh.
Grundfest, Harry, National Research Council Fellow, Johnson Foundation, University of Pennsylvania.
Hadley, Charles E., Assistant Professor of Biology, New Jersey State Teachers College.
Hahnert, William F., National Research Fellow, The Johns Hopkins University.
Ham, Arthur W., Instructor in Cytology, Washington University School of Medicine.

Hamburger, Rudolf T., Assistant in the Medical Clinic, University of Groningen, Holland.
Harnly, Morris H., Assistant Professor, New York University.
Hartline, H. K., Fellow, Johnson Foundation, University of Pennsylvania.
Harvey, Ethel Browne, Assistant in Biology, Washington Square College, New York University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Hayden, Margaret A., Assistant Professor of Zoölogy, Wellesley College.
Haynes, Florence W., Harvard University Medical School.
Haywood, Charlotte, Associate Professor of Physiology, Mount Holyoke College.
Heilbrunn, L. V., Associate Professor of Zoölogy, University of Pennsylvania.
Helwig, Edwin R., Instructor, University of Pennsylvania.
Henderson, Jean T., Lecturer, McGill University.
Henshaw, Paul S., Biophysicist, Memorial Hospital, New York City.
Hill, Samuel E., Assistant in Physiology, Rockefeller Institute.
Hoadley, Leigh, Professor of Zoölogy, Harvard University.
Hodge, Charles, Jr., Instructor in Zoölogy, University of Pennsylvania.
Hodge, Ruth M. Patrick, University of Virginia, Charlottesville, Virginia.
Hogue, Mary Jane, Instructor in Anatomy, Medical School, University of Pennsylvania.
Hook, Sabra J., Instructor in Biology, University of Rochester.
Hoppe, Ella N., Research Assistant, Division of Laboratories and Research, New York State Department of Health.
Horning, E. S., University of Sydney.
Howard, Evelyn, University of Pennsylvania.
Howe, H. E., Editor, Industrial and Engineering Chemistry.
Huettner, Alfred F., Associate Professor, Washington Square College, New York University.
Imai, Takeo, Assistant in Biology, Tohoku Imperial University, Sendai, Japan.
Irving, Laurence, Associate Professor of Physiology, University of Toronto.
Irwin, Marian, Associate, Rockefeller Institute.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.
Johlin, J. M., Associate Professor of Biochemistry, Vanderbilt University, School of Medicine.
Johnson, Duncan S., Professor of Botany, Johns Hopkins University.
Johnson, H. Herbert, Instructor, College of the City of New York.
Johnston, Robert L., Head of Research Division, Cleveland Clinic Foundation.
Kaufmann, Berwind P., Professor of Botany, University of Alabama.
Keefe, Rev. A. M., Rector, St. Norbert College.
Keil, Elsa M., Instructor in Zoölogy, New Jersey College for Women.
Kille, Frank R., Associate Professor of Biology, Birmingham-Southern College.
Kindred, James E., Associate Professor of Histology and Embryology, University of Virginia.
King, Robert L., Associate Professor, State University of Iowa.
Kirby-Smith, Henry T., Instructor in Anatomy, University of Pennsylvania.
Knower, Henry McE., Associate Professor of Anatomy, Albany Medical College.
Knowlton, Frank P., Professor of Physiology, Syracuse University, College of Medicine.
Kostir, Wencel J., Assistant Professor of Zoölogy, Ohio State University.
Lackey, James B., Professor of Biology, Southwestern.
Levine, Philip, Associate, Rockefeller Institute.
Lewis, Ivey F., Professor of Botany, University of Virginia.
Lillie, Frank R., Chairman of the Department of Zoölogy, University of Chicago.
Lillie, Ralph S., Professor of General Physiology, University of Chicago.
Loebel, Robert O., Fellow, Cornell University Medical College.
Lucas, Alfred M., Assistant Professor of Cytology, Washington University Medical School.

Lucas, Miriam Scott, Instructor in Cytology, Washington University Medical School.
Lucké, Balduin, Associate Professor of Pathology, University of Pennsylvania.
Lund, E. J., Professor of Physiology, University of Texas.
Lynch, Ruth Stocking, Instructor in Genetics, Johns Hopkins University.
McClung, C. E., Director, Zoölogical Laboratory, University of Pennsylvania.
McGlone, Bartgis, Instructor in Physiology, University of Pennsylvania.
McGoun, Ralph C., Jr., Instructor in Biology, Amherst College.
McGregor, James H., Professor of Zoölogy, Columbia University.
Margolin, Sydney, Graduate Student, Columbia University.
Mathews, Albert P., Carnegie Professor of Biochemistry, University of Cincinnati.
Mavor, James W., Professor of Biology, Union College.
Metz, Charles W., Professor, Johns Hopkins University.
Michaelis, L., Member, Rockefeller Institute for Medical Research.
Miller, Helen M., National Research Council Fellow, Johns Hopkins University.
Mitchell, Philip H., Professor of Physiology, Brown University.
Morgan, Ann H., Professor of Zoölogy, Mount Holyoke College.
Morgan, Lilian V., California Institute of Technology.
Morgan, T. H., Professor of Biology, California Institute of Technology.
Morgulis, Sergius, Professor of Biochemistry, University of Nebraska.
Morrill, Charles V., Associate Professor of Anatomy, Cornell University Medical College.
Morris, Helen S., Graduate Student, Columbia University.
Nabrit, S. Milton, Professor of Biology, Morehouse College.
Navez, Albert E., Lecturer in General Physiology, Harvard University.
Nelsen, Olin E., Instructor in Zoölogy, University of Pennsylvania.
Nicholas, Warren W., X-Ray Physicist, National Bureau of Standards.
Nonidez, José F., Assistant Professor of Anatomy, Cornell University Medical College.
Orr, Paul R., Instructor, University of Pennsylvania.
Osterhout, W. J. V., Member, Rockefeller Institute for Medical Research.
Packard, Charles, Assistant Professor of Zoölogy, Columbia University.
Papenfuss, George F., Student Assistant, Johns Hopkins University.
Parker, G. H., Professor of Zoölogy, Harvard University.
Parmenter, Charles L., Associate Professor of Zoölogy, University of Pennsylvania.
Parpart, Arthur K., Instructor in Physiology, University of Pennsylvania.
Patch, Esther M., Windsor, Vermont.
Payne, Fernandus, Head of Department of Zoölogy and Dean of Graduate School, Indiana University.
Payne, Nellie M., University of Pennsylvania.
Peebles, Florence, Professor of Biology, California Christian College.
Pinney, Mary E., Professor of Zoölogy, Milwaukee-Downer College.
Plough, Harold H., Professor of Biology, Amherst College.
Pollister, Arthur W., Instructor in Zoölogy, Columbia University.
Pollister, Priscilla Frew, Graduate Student, Columbia University.
Pond, Samuel E., Assistant Professor of Physiology, University of Pennsylvania.
Poole, James P., Professor of Evolution, Dartmouth College.
Raffel, Daniel, Fellow, Johns Hopkins University.
Redfield, Helen, California Institute of Technology.
Reese, Albert M., Head of Department of Zoölogy, West Virginia University.
de Renyi, George S., Associate Professor of Anatomy, University of Pennsylvania.
Reznikoff, Paul, Instructor in Medicine, Cornell University Medical College.
Richards, Alfred N., Professor of Pharmacology, University of Pennsylvania.
Richards, Oscar W., Instructor in Biology, Yale University.

Rijlant, Pierre, Professor of Human Physiology, University of Brussels.
Risley, Paul L., Instructor in Zoölogy, University of Michigan.
Root, Walter S., Assistant Professor of Physiology, College of Medicine, Syracuse University.
Rugh, Roberts, Instructor in Zoölogy, Hunter College.
Sayles, Leonard P., Instructor in Biology, College of the City of New York.
Schauffler, William G., Princeton, New Jersey.
Schmidt, Ida T. Genther, Research Fellow, Children's Hospital, Cincinnati, Ohio.
Schmidt, Leon H., Research Fellow, University of Cincinnati.
Schrader, Franz, Professor of Zoölogy, Columbia University.
Schrader, Sally Hughes, Columbia University.
Schultz, Jack, Investigator, Carnegie Institute of Washington.
Scott, Sister Florence Marie, Assistant Professor, Seton Hill College.
Sellmeyer, Bernard L., Head of Department and Professor of Biology, Loyola University.
Severinghaus, Aura E., Assistant Professor of Anatomy, Columbia University.
Sickles, Grace, Assistant Bacteriologist, New York State Department of Health.
Slifer, Eleanor H., National Research Council Fellow in Zoölogy, State University of Iowa.
Snook, Theodore, Instructor in Histology and Embryology, Cornell University.
Sonneborn, Tracy M., Research Associate, Johns Hopkins University.
Speidel, Carl C., Associate Professor of Anatomy, University of Virginia.
Spemann, Hans, Travelling Professor, Rockefeller Foundation.
Steinbach, Henry Burr, Instructor in Zoölogy, University of Pennsylvania.
Stewart, Dorothy R., Assistant Professor of Biology, Skidmore College.
Stockard, Charles R., Professor of Anatomy, Cornell University Medical College.
Street, Sibyl, 29 Jewett Place, Utica, New York.
Strong, Oliver S., Professor of Neurology and Neuro-Histology, Columbia University.
Sumwalt, Margaret, Assistant Professor of Physiology, Woman's Medical College of Pennsylvania.
Taft, Charles H., Jr., Associate Professor of Pharmacology, University of Texas, Medical School.
Tashiro, Shiro, Professor of Biochemistry, University of Cincinnati.
Taylor, Wm. Randolph, Professor of Botany, University of Michigan.
Tittler, Irving A., Assistant in Zoölogy, Columbia University.
Titus, Charles P., East Orange, New Jersey.
Tohyama, Guzo, Assistant Professor, Tokyo Imperial University.
Turner, John P., Instructor in Zoölogy, University of Minnesota.
Tyler, Albert, Instructor in Embryology, California Institute of Technology.
Unger, W. Byers, Assistant Professor of Zoölogy, Dartmouth College.
Van Slyke, E., Instructor, University of Pittsburgh.
Vicari, Emilia M., Research Associate in Anatomy, Cornell University Medical College.
Walker, Ruth I., Instructor in Botany, University of Wisconsin.
Warren, Howard C., Professor of Psychology, Princeton University.
Whedon, Arthur D., Professor of Zoölogy and Head of Department of Zoölogy and Physiology, North Dakota Agricultural College.
Whitaker, D. M., Assistant Professor of Zoölogy, Columbia University.
Whiting, Anna R., Professor of Biology, Head of Department, Pennsylvania College for Women.
Whiting, P. W., Associate Professor of Zoölogy, University of Pittsburgh.
Wieman, H. L., Professor of Zoölogy, University of Cincinnati.
Wilson, Edmund B., Professor Emeritus, Columbia University.
Witschi, Emil, Professor of Zoölogy, State University of Iowa

Wodehouse, Roger P., Director of the Biological Laboratory, The Arlington Chemical Co.
Wolf, E. Alfred, Assistant Professor of Zoölogy, University of Pittsburgh.
Woodward, Alvalyn E., Assistant Professor, University of Michigan.
Young, Roger Arliner, Assistant Professor of Zoölogy, Howard University.

Beginning Investigators

Adams, Edgar M., Laboratory Assistant, University of Cincinnati.
Apgar, Grace M., University of Pennsylvania.
Atlas, Meyer, Assistant in Embryology, Columbia University.
Auringer, Jack, Graduate Student, Detroit City College.
Beck, Lyle V., Graduate Assistant in Physiology, New York University.
Benkert, Joseph M., Science Director, Ambridge High School.
Benkert, Lysbeth Hamilton, Instructor, Pennsylvania College for Women.
Bostian, C. H., Assistant Professor of Zoölogy, North Carolina State College.
Bradway, Winnefred, Assistant in Biology, Washington Square College, New York University.
Buchheit, J. Robert, Graduate Assistant in Zoölogy, University of Illinois.
Cable, Raymond M., Teaching Assistant, New York University.
Carlson, J. Gordon, Demonstrator in Biology, Bryn Mawr College.
Chen, T. T., Instructor and Graduate Student, University of Pennsylvania.
Chor, Herman, Research Fellow, Washington University Medical School.
Cline, Elsie, Graduate Student, Johns Hopkins University.
Cohen, Bernard M., Student, Johns Hopkins University.
Corson, Samuel A., University of Pennsylvania.
Costello, Donald P., Instructor in Zoölogy, University of Pennsylvania.
Croasdale, Hannah T., Scientific Staff of Biological Abstracts, University of Pennsylvania.
Dan, Katsuma, University of Pennsylvania.
Daugherty, Kathryn, Research Assistant, University of Pennsylvania.
Davis, J. F., Instructor in Zoölogy, University of Pennsylvania.
Dearing, William H., Student, University of Pennsylvania Medical School.
Eastlick, Herbert L., Part Time Assistant, Washington University.
Field, Madeleine E., Research Fellow in Physiology, Harvard School of Public Health.
Gilmore, Kathryn, Graduate Student, University of Pittsburgh.
Gray, Nina E., Assistant in Zoölogy, University of Wisconsin.
Green, David E., Assistant, Washington Square College, New York University.
Hamburgh, Morton, Johns Hopkins University Medical School.
Heiss, Elizabeth, Instructor in Histology, Purdue University.
Hill, Edgar S., University of Cincinnati.
Hitschler, William J., Research Worker, University of Pennsylvania.
Ishii, Kenjiro, Graduate Student, University of Cincinnati.
Jackson, John R., Graduate Assistant in Botany, University of Missouri.
Jones, Edgar P., Instructor, University of Pittsburgh.
Katz, Jacob D., Graduate Student, New York University.
Keltch, Anna K., Lilly Research Laboratories.
Kidder, George W., Graduate Student, Columbia University.
Kinney, Elizabeth T., Assistant in Zoölogy, Barnard College.
Krajnik, Bohumil, Research Assistant, University at Bratislava, Czechoslovakia.
Lambert, Elizabeth F., Technician, Harvard University Medical School.
Lundstrom, Helen M., Graduate Student, University of Pennsylvania.
McQuesten, Barbara, 14 Still Street, Brookline, Massachusetts.
Machella, Thomas E., Assistant Instructor, University of Pennsylvania.
Mann, D. R., Graduate Assistant, Duke University.
Marshak, Alfred, Student, Bussey Institution.

Marsland, Douglas A., Assistant Professor of Biology, Washington Square University, New York University.
Medaris, Don, Student, DePauw University.
Meltzer, Adolph, Student, Cornell University Medical College.
Mendelson, Emanuel S., Research Technician, University of Pennsylvania.
Michaelis, Eva, 1185 Park Avenue, New York City.
Morris, Samuel, Instructor in Zoölogy, University of Pennsylvania.
Nicoll, Paul A., Part Time Assistant, Washington University.
Oltmann, Clara, Student, Columbia University.
Ormsby, Andrew A., 14209 Winthrop Avenue, Detroit, Michigan.
Parks, Mark E., Assistant in Biology, New York University.
Pierce, Madelene E., Graduate Student, Radcliffe College.
Powers, Phil B., Instructor in Zoölogy, University of Pennsylvania.
Robinson, Ellis J., Graduate Assistant, New York University.
Root, Clinton W., Assistant in Biochemistry, Princeton University.
Schechter, Victor, College of the City of New York.
Schuett, J. F., Assistant in Zoölogy, University of Chicago.
Schweitzer, Morton D., Assistant in Zoölogy, Columbia University.
Shapiro, Herbert, Assistant in Zoölogy, Columbia University.
Shaw, C. Ruth, Faculty Member, Kent State College.
Shore, Agnes, Instructor in Chemistry, Bellevue Medical College, New York University.
Sichel, Ferdinand J., Assistant Instructor, Washington Square College, New York University.
Southwick, Walter E., Student, Harvard University.
Speicher, B. R., Graduate Assistant, University of Pittsburgh.
Stancati, Milton F., Graduate Assistant, University of Pittsburgh.
Sturdivant, H. P., Instructor, Columbia University.
Torvik, Magnhild, Graduate Assistant, University of Pittsburgh.
Townsend, Grace, Fellow, University of Chicago.
Twyeffort, Louis H., Instructor in Biology, Princeton University.
Wald, George, Research Assistant, Columbia University.
Walker, Paul A., Student, Bowdoin College.
Weiner, Eleanor, Graduate Student, University of Pennsylvania.
Weisman, Maxwell N., Fellow, College of the City of New York.
Welty, Carl, Assistant Professor of Biology, Parsons College.
Wilde, Mary H., Graduate Assistant in Botany, New Jersey College for Women.
Williams, Mary E., 37 Addington Road, Brookline, Massachusetts.
Winsor, Agnes A., Volunteer Associate in Biology, School of Hygiene, Johns Hopkins University.
Winsor, Charles P., Associate in Biology, School of Hygiene and Public Health, Johns Hopkins University.

Research Assistants

Alderman, Evangeline, Graduate Assistant, Wellesley College.
Armstrong, Eleanor F., Cornell University Medical College.
Ashkenaz, David M., Assistant in Biology, New York University.
Bailey, Sarah W., Student, Radcliffe College.
Boyd, Milford J., Assistant Graduate Student, University of Cincinnati.
Butt, Charles, Research Assistant, Princeton University.
Carabelli, A. Albert, Medical Student, University of Pennsylvania.
Coldwater, K. B., Instructor in Zoölogy, University of Missouri.
Cole, Robert, 45 King Street, Oberlin, Ohio.
Davis, James E., Laskar Fellow and Research Assistant in Medicine, University of Chicago.

DuBois, Anne Marie, Research Assistant, Carnegie Institution of Washington.
Erlanger, Margaret, Harvard University Medical School.
Eyre, Sarah W., Research Assistant, Long Island University.
Fowler, J. R., Research Assistant, University of Chicago.
Francis, Dorothy S., Research Assistant in Biophysics, Memorial Hospital, New York City.
Friedheim, Ernst A. H., Rockefeller Institute.
Graham, Clarence H., National Research Fellow, Johnson Foundation, University of Pennsylvania.
Graubard, Marc A., National Research Council Fellow.
Harnly, Marie L., Assistant in Biology, New York University.
Harryman, Ilene, Lilly Research Laboratories.
Henshaw, Christine T., Assistant Biophysicist, Memorial Hospital, New York City.
Hilsman, Helen, Assistant, University of Pittsburgh.
Holt, Helen, Assistant, Washington Square College, New York University.
Homès, Marcel V. L., Assistant in Botany, University of Brussels.
Larrabee, Martin G., Student, Harvard College.
Parks, Elizabeth K., Instructor in Histology and Embryology, Boston University Medical School.
Parpart, Ethel R., 230 Nassau Street, Princeton, New Jersey.
Rempe, Alois E., Washington University Medical School.
Schluger, Jack, Research Assistant, New York University.
Schmuck, M. Louise, Research Assistant, Carnegie Institution.
Scott, Allan C., Graduate Assistant in Zoölogy, University of Pittsburgh.
Sell, James P., Student, Oberlin College.
Smith, Helen Berenice, Research Assistant, Carnegie Institution of Washington.
Smith, M. Doreen, Research Assistant, University of Toronto.
Smith, Suzanne G., Research Assistant, University of Missouri.
Stokes, Julia C., Research Assistant, Washington University.
Tang, Pei-Sung, Research Fellow, Harvard University.
Tocker, Albert M., Research Assistant, Washington University Medical School.
Tyler, Betty S., California Institute of Technology.
Van Alstyne, Margaret A., Assistant, Harvard University Medical School.
Wade, Lucille W., Lilly Research Laboratories.
Wilson, Hildegard N., Teaching Fellow, New York University and Bellevue Hospital Medical College.
Young, Saul B., Technician, Rockefeller Institute.

Students

BOTANY

Altland, Clair S., American University.
Andrew, Barbara R., Assistant Instructor in Botany, University of Alabama.
Bryan, Hilah F., Smith College.
Hunt, William, Student, Southwestern.
Jackson, John R., Graduate Assistant in Botany, University of Missouri.
Moore, Caroline, Student, University of Pennsylvania.
Perry, Lily M., Graduate Student, Washington University.

EMBRYOLOGY

Alderman, Evangeline, Graduate Assistant, Wellesley College.
Alexander, Lloyd E., Instructor, Fisk University.
Alexanderson, Amelie M., Student, Bryn Mawr College.

Boone, Eleanor S., Instructor in Zoölogy, Mills College.
Buchheit, J. Robert, Assistant in Zoölogy, University of Illinois.
Buck, Margaret A., Student, University of Maine.
Cable, Raymond M., University Fellow, New York University.
Carlson, J. Gordon, Graduate Student, University of Pennsylvania.
Chase, Hyman Y., Graduate Student, Howard University.
Chen, Hsin T., Student, Harvard University.
Coulter, Edith A., Student, Goucher College.
Denny, Martha, Radcliffe College.
Derrickson, Mary B., Graduate Assistant, Syracuse University.
Dick, George A., Professor of Animal Industry, University of Pennsylvania.
Kaston, Benjamin J., Graduate Student, Yale University.
Magruder, Samuel R., Graduate Assistant in Zoölogy, University of Cincinnati.
Newcomer, A. Virginia, Radcliffe College.
Oppenheimer, Jane M., Student, Bryn Mawr College.
Plyler, Phyllis V., Goucher College.
Price, John B., Graduate Student, Stanford University.
Sawyer, Elizabeth L., 29 Elm Street, Bangor, Maine.
Sell, James P., Oberlin College.
Shea, Margaret M., Graduate Assistant, Wellesley College.
Smith, Wilson F., Jr., Student, Cornell University Medical College.
Townsend, Grace, Fellow, University of Chicago.
Walker, Paul A., Bowdoin College.
Weed, Milton R., Wesleyan University.
Woodruff, Beth H., Graduate Assistant, Western Reserve University.
Woodside, Gilbert L., Assistant in Embryology Laboratory, DePauw University.

PHYSIOLOGY

Barney, Raymond L., Professor of Biology, Middlebury College.
Beck, Lyle V., Graduate Assistant, New York University.
Fisher, Kenneth C., Student, Acadia University.
Gaetjens, Laura C., Elmira College.
Green, David E., Teaching Fellow, New York University.
Heiss, Elizabeth M., Instructor in Biology, Purdue University.
Lundstrom, Helen M., Graduate Student, University of Pennsylvania.
McQuesten, Barbara, 14 Still Street, Brookline, Massachusetts.
Michaelis, Eva, 1185 Park Avenue, New York City, New York.
Moore, Elinor, Student, University of Pennsylvania.
Morgan, Isabel M., Student, Stanford University.
Noll, Clarence I., Laboratory Assistant in Chemistry, Trinity College.
Prosser, C. Ladd, Graduate Student and Assistant, Johns Hopkins University.
Reid, Marion A., Instructor in Physiology, Boston University School of Medicine.
Scherp, Henry W., Assistant, Rockefeller Institute.
Sweetman, Harvey L., Assistant Professor of Entomology, Massachusetts State College.
Willard, William R., Student, Yale School of Medicine.

PROTOZOÖLOGY

Adell, James C., Assistant, Teachers College, Columbia University.
Auringer, Jack, College of City of Detroit.
Brown, Rebecca R., Columbia University.
Burr, Edith Rogers, Columbia University.
Carpenter, Helena Jane, Ohio Wesleyan University.
Dee, M. Barbara, Assistant, Jamaica Plain High School.

Ericson, Alma L., Critic Teacher in Biology, Hunter College Model School.
Eskridge, Lydia C., Technical Assistant, Johns Hopkins University.
Fenton, Frances E., Teacher of General Science, Connecticut College.
Henderson, Lillian O., Instructor in Biology, H. Sophie Newcomb College.
Hutchings, Lois M., Teacher of High School Biology, Newark, New Jersey.
Ickes, Marguerite, Teacher of Biology, Lincoln High School, Cleveland, Ohio.
James, Miriam E., Science Teacher in High School, Gloucester, Massachusetts.
Ormsby, Andrew A., College of City of Detroit.
Sperry, Helen O., Teacher of Biology, Cathedral School of St. Mary.
Stewart, Paul A., University of Rochester.
Watkins, Evelyn G., Student, Vassar College.

INVERTEBRATE ZOÖLOGY

Aguago, Carlos S., Assistant Professor of Biology, University of Havana.
Anthony, Elizabeth S., Graduate Student, Brown University.
Bachrach, Josephine E., Student, Vassar College.
Baker, E. G. Stanley, Student Assistant, DePauw University.
Barron, Donald H., Graduate Student, Yale University.
Belcher, Jane C., Colby College.
Brewster, James R., Biological Production, University Film Foundation.
Chase, Hyman Y., Graduate Student, Howard University.
Chen, Hsin T., Student, Harvard University.
Chinn, Mary Priscilla, Goucher College.
Clark, Adele F., Student, Tufts College.
Clark, Jean M., Student, Wilson College.
Clausen, Ralph G., Instructor, Union College.
Cohen, Bernard M., Assistant in Zoölogy, Johns Hopkins University.
Corey, H. Irene, Research Assistant and Secretary, University of Pennsylvania.
Croly, John T., Student, Dartmouth College.
Dimick, Helen, Student, Wellesley College.
Drew, R. W., Student, Wesleyan University.
Drugg, Helen, Teacher, University of Vermont.
Eastlick, Herbert L., Graduate Assistant in Zoölogy, Washington University.
Ellis, Lola M., Assistant in Biology Department, Southwestern.
Fish, Harold S., Student Assistant, Harvard University.
Forhan, Laura Jo, Student, University of Montana.
Fuchs, Barrett, Student Assistant in Biology, American University.
Gerstell, Richard, Student, Dartmouth College.
Glidden, Dorothy P., Smith College.
Godwin, Melvin, Student, DePauw University.
Hegner, Isabel, Student, Radcliffe College.
Hetrick, Lawrence A., Jr., American University.
Howard, John W., Student, Hamilton College.
Hussey, Kathleen L., Fellow in Zoölogy, Ohio Wesleyan University.
Jefferson, Margaret D., Graduate Student, University of Pennsylvania.
Johnson, Arlene C., Student, Wheaton College.
Jones, E. R., Lecturer in Biology, Dalhousie University.
Kilgore, Byron, Jr., Butler University.
Kramer, Theodore C., Research Assistant in Biology, Western Reserve University.
Langstroth, Muriel A., Dalhousie University.
Mann, Donald R., Investigator, Duke University.
Metzner, Jerome, Fellow, College of the City of New York.
Moment, Gairdner B., Graduate Student, Yale University.

Nicoll, Paul A., Graduate Assistant, Washington University.
Pomerat, Charles M., Assistant in Biology, Clark University.
Raye, William H., Jr., Amherst College.
Rosenbaum, Louise, Laboratory Assistant, University of Pennsylvania.
Rountree, Katherine E., Instructor in Biology, Wesleyan College.
Sanders, Rosaltha, Student, Yale University.
Smith, Osgood R., Hamilton College.
Solberg, Archie N., Instructor in Zoölogy, North Dakota Agricultural College.
Stewart, Paul A., University of Rochester.
Thomas, Thurlo B., Graduate Assistant, Oberlin College.
Warters, Mary, Assistant Professor of Zoölogy, Centenary College.
Westkaemper, Sister Remberta, Head of Department of Biology, College of St. Benedict.
Willard, William R., Student, Yale School of Medicine.
Wismer, Virginia, Assistant, University of Pennsylvania.
Young, George D., Student, Acadia University.

3. TABULAR VIEW OF ATTENDANCE

	1927	1928	1929	1930	1931
Investigators—Total	294	323	329	337	362
Independent	209	217	234	217	236
Under Instruction	57	81	71	87	83
Research Assistants	28	25	24	33	43
Students—Total	141	133	125	136	125
Zoölogy	57	57	53	56	55
Protozoölogy	17	16	15	14	17
Embryology	32	29	28	27	29
Physiology	19	15	17	23	17
Botany	16	16	12	16	7
Total Attendance	435	456	454	473	487
Less Persons registered as both Students and Investigators	1	2	10	14	20
	434	454	444	459	467
Institutions Represented—Total	111	111	123	126	137
By Investigators	89	80	96	95	102
By Students	63	66	64	71	68
Schools and Academies Represented					
By Investigators	1	1	–	–	–
By Students	4	1	1	4	4
Foreign Institutions Represented					
By Investigators	15	13	30	7	8
By Students	8	8	3	2	1

4. SUBSCRIBING AND COÖPERATING INSTITUTIONS, 1931

Acadia University
American University, Washington, D. C.
Amherst College
Barnard College
Bowdoin College
Brown University
Bryn Mawr College
Butler College
C. R. B. Educational Foundation
California Institute of Technology
Carnegie Institution, Cold Spring Harbor
Carnegie Institution of Washington
College of St. Benedict
Columbia University
Cornell University
Cornell University Medical College
Dalhousie University
Dartmouth College
DePauw University
Duke University
Elmira College
General Education Board
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Howard University
Hunter College
Indiana University
Industrial & Engineering Chemistry, of the American Chemical Society
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly & Co.
Long Island University
Loyola University
Memorial Hospital of New York City
Morehouse College
Mount Holyoke College
National Research Council
New York State Department of Health
New York University
Oberlin College
Pennsylvania College for Women
Princeton University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Seton Hill College
Smith College
Sophie Newcomb College
Southwestern
Syracuse University
Tufts College
Union College
United States Department of Agriculture
University of Buffalo
University of Chicago
University of Chicago Medical School
University of Cincinnati
University of Illinois
University of Iowa
University of Minnesota
University of Missouri
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Texas
University of Virginia
University of Wisconsin
Vanderbilt University Medical School
Vassar College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Western Reserve University
West Virginia University
Wheaton College
Wilson College
Wistar Institute of Anatomy and Biology
Yale University

SCHOLARSHIP TABLES

Lucretia Crocker Scholarships for Teachers in Boston.

Scholarship of $100 supported by a friend of the Laboratory since 1898.

The Edwin S. Linton Memorial Endowment of Washington and Jefferson College, given by Edwin Linton and Margaret Brownson Linton in memory of their son, member of the class of 1913, who gave his life in France during the World War. The endowment amounts to $2,500, and the income therefrom is to be used to encourage study and research at the Marine Biological Laboratory, Woods Hole, Massachusetts.

The Bio Club Scholarship of the College of the City of New York.

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5. EVENING LECTURES, 1931

Tuesday, June 23 Dr. Leonor Michaelis	“Theory of the Heme Pigments as Oxygen Carriers and as Oxidation Catalysts.”
Friday, July 3 Dr. E. B. Wilson	“The Central Bodies.” Illustrated by Original Photomicrographs.
Friday, July 10 Dr. G. H. Parker	“Humoral Agents in Nervous Activities with Special Reference to Chromatophores.”
Friday, July 17 Dr. Eliot R. Clark	“The Microscopic Study of Cells and Tissues in the Living Mammal.”
Friday, July 24 Dr. T. H. Morgan	“The Marine Laboratories of the World and Their Work.”
Friday, July 31 Dr. H. Spemann	“Experiments on the Amphibian Egg.”
Friday, August 7 The Reynold A. Spaeth Memorial Lecture, delivered by Dr. Ross G. Harrison	“Problems and Methods of Experimental Embryology.”
Friday, August 14 Dr. F. L. Hisaw	“The Corpus Luteum and Anterior Lobe Hormones and Their Physical Inter-relationships.”
Friday, August 21 Dr. Charles R. Stockard	“An Experimental Dog Farm for the Study of Form and Type.”
Wednesday, August 26 Dr. E. D. Congdon	“Some Impressions, Racial and Cultural, of the Siamese.”

Friday, August 28

Dr. J. H. McGregor .... "Motion Pictures Taken in the Belgian Congo and the Cameroon by the African Expedition (1929–30) of Columbia University and the American Museum of Natural History."

Friday, September 4

Dr. Bradley M. Patton .... "Micro-moving Pictures Applied to the Study of the Living Embryo."

SPECIAL LECTURES AND MOTION PICTURES

Saturday, July 18

Motion Pictures .... "Modern Studies of Sulphur."
"Monel Metal."

Thursday, July 23

Dr. A. E. Navez .... "Geotropism in Plants."

Mr. Leonard Craske .... "The Art and Uses of Color Photography."

Thursday, August 20

Motion Pictures .... "Cleaving Eggs of Echinarachnius and Arbacia." (Dr. Henry J. Fry)
"The Redistribution of Granules in Centrifuged Arbacia Eggs." (Dr. E. N. Harvey)
"Arterio-venous Anastomoses." (Dr. E. R. Clark)
"The Function of the Intestinal Villi." (Dr. F. Verzár—comments by Dr. W. E. Garrey)

6. SHORTER SCIENTIFIC PAPERS, 1931

Tuesday, June 30

Dr. G. S. Dodds .... "Osteoclasts and Chondroclasts."

Dr. A. W. Pollister .... "The Architecture of the Liver Cells of Amphiuma."

Dr. G. H. Parker .... "Passage of Sperms and Eggs through the Mammalian Oviduct."

Tuesday, July 7

Dr. A. C. Redfield .... "Effect of Hydrogen Ion Concentration and Salt Concentration on the Oxygen Dissociation Constant of Hemocyanin."

Dr. Laurence Irving .... "The Carbon Dioxide Dissociation Curve of Living Mammalian Muscle."

Dr. E. N. Harvey .... "Photo-electric Records of Animal Luminescence."

Tuesday, July 14

Dr. H. H. Plough .... "Some Observations on Self Sterility in Styela."

Dr. R. Chambers .... “Evidence of a Direct Action of the Nucleus on the Cytoplasm in Tissue Cultures.”
Dr. A. F. Huettner .... “Genetic Continuity of the Central Bodies.”

Tuesday, July 21
Dr. S. Morgulis .... “The Chemistry of Bone Ash.”
Dr. J. M. Johlin .... “The Enolization of Gelatin by Neutral Salts.”
Dr. E. S. Guzman Barron .... “Oxidations Produced by Gonococci.”
Dr. Shiro Tashiro and
Mr. L. H. Schmidt .... “Bile Salts.”

Monday, July 27
Dr. J. P. Turner .... “The Fibrillar System in Euplotes.”
Dr. Daniel Raffel .... “Types of Variation Produced by Conjugation in Paramecium aurelia.”
Dr. Ruth S. Lynch .... “Effects of Conjugation in a Number of Clones of Paramecium aurelia.”
Dr. T. M. Sonneborn .... “Crossing Diverse Clones of Paramecium aurelia.”

Wednesday, July 29
Under the Auspices of the Society of Cellular Biology
Dr. Balduin Lucké .... “The Mechanism of Bacteriotropin Action.”
Dr. M. H. Jacobs and
Dr. A. K. Parpart .... “Is the Permeability of the Erythrocyte to Water Decreased by Narcotics?”
Dr. L. V. Heilbrunn .... “The Action of the Common Cations on the Protoplasmic Viscosity of Amoeba.”
Dr. R. Chambers .... “The Formation of Ice Crystals in the Protoplasm of Various Cells.”

Tuesday, August 4
Dr. E. F. DuBois .... “Surface Temperature and the Radiation of Heat from the Human Body.”
Dr. Pierre Rijlant .... “Oscillographic Study of the Cardiac Ganglion of Limulus polyphemus.”
Dr. D. M. Whitaker .... “The Change in Rate of Oxygen Consumption at Fertilization of the Eggs of Chaetopterus, Cumingia, Nereis, Arbacia and Fucus.”
Dr. R. W. Gerard .... “Phosphocreatin in Nerve in Relation to Activity.”

Tuesday, August 11
Dr. W. H. F. Addison .... “Aquatic Mammals—A Description of a Special Cell Type in the Cerebellum.”

Dr. C. C. Speidel	"Living Nerve Sprouts."
Dr. J. E. Kindred	"Histologic Effects of Ligation on the Vasa of the Spleen of the Albino Rat."
Dr. G. S. deRenyi	"The Effect of Radium Irradiation upon the Ovaries of the Albino Rat."
Tuesday, August 18	
Dr. Helen B. Smith	"Genetic Studies on Selective Segregation of Chromosomes in Sciara."
Dr. P. W. Whiting	"Local and Correlative Gene Effects in Mosaics of Habrobracon."
Dr. C. B. Bridges	"Specific Modifiers in Drosophila melanogaster."
Tuesday, August 25	
Dr. Paul S. Henshaw	"Recovery from X-ray Effects as Observed in Arbacia Eggs."
Mr. Ware Cattell	"The Reaction of the Fundulus Ovum to the Direct Electric Current."
Dr. E. A. Wolf and Dr. H. H. Collins	"The Effect of Ultra-violet Radiation upon the Color Pattern of Triturus."
Dr. G. H. Parker	"The Discharge of Nematocysts."
Dr. Dmitry N. Borodin	"Biological Spectrum and M-rays." (Motion pictures)
Tuesday, September 1	
Dr. Albert Tyler	"Artificial Parthenogenesis in the Eggs of the Pacific Coast Echiuroid, Urechis caupo."
Dr. Paul S. Galtsoff	"Specificity of Sexual Reactions in the Genus Ostrea."
Dr. K. B. Coldwater	"The Effect of Sulphydryl Compounds upon Regenerative Growth."
Dr. N. A. Cobb	"The Use of Live Nemas in Zoological Courses in Schools and Colleges."
Thursday, September 3	
Dr. Charlotte Haywood and Dr. Walter S. Root	"The Cleavage Rate of the Arbacia Egg in the Presence of Carbon Dioxide and Bicarbonate."
Dr. Arthur K. Parpart and Dr. M. H. Jacobs	"The Action of Acetic Acid and Its Sodium Salt on the Cleavage of Arbacia Eggs."
Mr. K. Dan	"Cataphoretic Studies of Marine Eggs."
Dr. Kenneth Cole	"Surface Forces of the Arbacia Egg."
Dr. E. N. Harvey	"The Tension at the Surface of Arbacia Eggs, Determined by Centrifugal Force."

Dr. Ethel Browne Harvey	"Development of Arbacia Half-Eggs Produced by Centrifugal Force."
Dr. Balduin Lucké	"Osmotic Properties of 'Fragments' of Arbacia Eggs Obtained by Centrifugal Force."
Dr. M. H. Jacobs and Dr. Dorothy R. Stewart	"A Method for the Quantitative Measurement of Cell Permeability."
Dr. Dorothy R. Stewart and Dr. M. H. Jacobs	"The Effect of Fertilization on the Permeability of the Arbacia Egg to Ethylene Glycol."
Dr. Miriam Scott Lucas	"Recent Observations upon a Type of Fission Undescribed for Ciliates."
Dr. E. C. Cole	"Selective Intra-Vitam Staining of Specific Elements in the Integument of the Squid."
Dr. C. C. Speidel	"Types of Nerve Regeneration, as Revealed by Prolonged Observation of Individual Fibers in Living Frog Tadpoles."
Dr. H. H. Johnson	"Centrioles and Other Cytoplasmic Bodies in Living Cells of Gryllids."
Dr. P. W. Whiting	"Genetic Results in Habrobracon Bearing on Maturation and Fertilization."
Mr. L. V. Beck and Mr. D. E. Green	"Oxidation-reduction Potentials of Cytolyzed and Intact Echinoderm Eggs."
Dr. Eric G. Ball	"Hemolysis of Fish Erythrocytes by an Impurity in Sodium Chloride."
Dr. G. H. A. Clowes, Dr. I. H. Page and Mr. H. A. Shonle	"On the Contrasting Cytolytic Effects Exerted by Soaps of the Type of Sodium Ricinoleate and Sodium Oleate at Different H Ion Concentrations and the Relation of These Effects to the Oil-water Interfacial Tensions Exerted by the Soaps in Question."
Miss Anna Keltch, Miss Ilene Harryman and Dr. G. H. A. Clowes	"Influence of H Ion Concentration on the Anesthetic Value of a Series of General and Local Anesthetics and Hypnotics."
Mr. S. A. Corson	"The Action of Acid and Alkali on the Protoplasmic Viscosity of Amoeba dubia."

Mr. H. B. Steinbach	"The Effect of Salts on the Injury Current of Scallop Muscle."
Dr. Robert Chambers and Mr. D. A. Marsland	"The Action of the Common Salts on the Protoplasm of the Echinoderm Egg."
Mr. Morris Belkin	"Capping of Oils on Protoplasmic Surfaces."

Demonstrations

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Dr. C. B. Bridges	"Apparatus and Designs for Raising Drosophila."
Dr. E. R. Clark, Mrs. E. L. Clark, Dr. H. T. Kirby-Smith and Dr. W. J. Hitschler	"Living Tissues as Seen in Transparent Chambers Introduced into the Rabbit's Ear."
Dr. E. C. Cole	"Selective Intra-Vitam Staining of Specific Elements in the Integument of the Squid."
Dr. Kenneth Cole	"An Egg Crusher."
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Warren, Prof. Howard C., Princeton University, Princeton, N. J.

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WILSON, DR. J. W., Brown University, Providence, R. I.
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YOUNG, DR. B. P., Cornell University, Ithaca, N. Y.
YOUNG, DR. D. B., University of Maine, Orono, Me.
ZELENY, DR. CHARLES, University of Illinois, Urbana, Ill.

THE REACTION OF THE ERYTHROCYTES OF VERTEBRATES, ESPECIALLY FISHES, TO VITAL DYES

ALDEN B. DAWSON

(*From the Zoölogical Laboratories, Harvard University, and the Marine Biological Laboratory, Woods Hole, Massachusetts*)

In the erythrocytes of most vertebrates, but especially of fishes and amphibians, discrete granules are characteristically present. In the urodele, *Necturus,* bipolar clusters of such granules are regularly found in mature red blood cells. They are visible in fresh preparations, demonstrable as basophilic bodies with Wright or Giemsa staining, are blackened with osmic acid and silver salts, and are stained with iron hæmatoxylin after Helly fixation. Accordingly, there is no doubt that they are preëxistent structures and are not induced in supravital preparations by the action of the dyes.

However, secondary granules may also appear in such cells; the concentration of the dye, age of the preparation, brilliancy of illumination, and increase in temperature being effective as formative factors, influencing the rate and manner of their appearance (Dawson, 1928, 1929, 1930). Moreover, with higher concentrations of dye, the red cells may also exhibit elaborate patterns of reticulation. The genetic relation of both types of granules, preëxisting and induced, to the reticulated substance is an unsettled question. Morphologically there is no distinction between the two types and both are frequently enclosed in the reticular filaments. The reticulation pattern is apparently derived, through a reaction with the vital dye, from the basophilic substance which occurs diffusely in erythrocytes, and secondary granules are regarded by some as the same substance in a different form.

The literature on this subject is voluminous and many different views are advanced regarding the significance of the protoplasmic constituents reacting with vital dyes. First of all, the ability to react with a given vital dye may not be a specific characteristic of a single substance within the cytoplasm. Again, in many instances, the materials reacting with vital dyes are not always readily demonstrated by other technical methods. *Necturus* erythrocytes appear to be exceptionally favorable in this respect. Moreover, the amount of material reacting with vital dyes appears to decrease gradually as the erythrocytes differentiate, but the acquisition of hemoglobin in a given species proceeds only up to a certain stage at which the cell is said to be mature. Since the erythrocytes in different vertebrates do not attain at maturity the same relative degree of differentiation, the picture in supravital preparations is subject to great variation; the individual changes which occur in the maturation of the red blood cells may apparently proceed at different rates and to different degrees in different species. Accordingly, the relative concentration of hemoglobin within the erythrocytes of different species appears to be to some extent independent of the amount and distribution of material reacting with vital dyes. These are only some of the factors which complicate the picture and make generalizations almost impossible. Coupled with these there is also the lack of complete information about the stages of maturation of these cells in many species. In other cases, too, findings by one method have not been adequately checked and confirmed by other technics.

Various theories of the origin and nature of the vitally-stained bodies have been advanced. Several earlier workers suggested a nuclear or nucleolar origin (Giglio-Tos, 1896; Jolly, 1903; Sabrazès et Muratet, 1908), while others have apparently confused them with centrosomes (von Apáthy, 1897; Bremer, 1895; Dehler, 1895; Eisen, 1897–1899; Golgi, 1920) when demonstrated by non-vital staining methods such as silver nitrate impregnation or staining with iron hæmatoxylin. They were also identified as intracellular parasites, but this view was soon dropped (Sabrazès et Muratet, 1900).

At present the vital granules are generally regarded as of cytoplasmic origin. Giglio-Tos (1896) suggested that they represented hemoglobin-forming substances and Yoffey (1929) is inclined to favor this view. Several modern investigators (Ferrari, 1930; Villa, 1930; Knoll, 1931) have advanced evidence in favor of an intranuclear origin of hemoglobin, and this interpretation accordingly would support the old hypothesis of Giglio-Tos that vital granules arise as nuclear emissions and are concerned in the elaboration of hemoglobin. On the basis of the reaction of these bodies with silver salts and osmic acid, they have been regarded as possibly homologous with the Golgi apparatus—dictyosomes (Bhattacharya and Brambell, 1925; Dawson, 1928, 1930; Dornesco and Steopoe, 1930*a*, 1930*b*; Urtubey, 1927). Nittis (1930) described a surface granule which in his opinion might be interpreted as the point of separation of two daughter cells or as a part of a trophospongial system. Nittis' view seems untenable, as the single granule is intracellular and may frequently exhibit Brownian motion (Dawson, 1931). Many tend to class all vital granules as artifacts (Chlopin, 1927; Weidenreich, 1903), failing to distinguish sharply between preëxistent and induced bodies (Beams, 1930).

The number and distribution of vital granules in the erythrocytes of vertebrates vary during the differentiation of the cell. Usually they are not present in very young cells and are reduced or even disappear in old cells. In the intermediate stages they are frequently numerous and conspicuous. In the mature cells of different vertebrates in which they still persist, vital granules frequently occur in rather definite numbers and have a characteristic position within the cell. The more common types of distribution are single unipolar; multiple, clustered unipolar; multiple, clustered bipolar; multiple perinuclear; and multiple scattered or diffuse (Dawson and Charipper, 1929; Dawson, 1930, 1931).

The number and distribution of vital granules in the erythrocytes of cyclostomes and fishes vary considerably. They were apparently recognized as early as 1896 by Giglio-Tos, who observed them in the blood cells of the lamprey and called attention to the striking Brownian movements they exhibited. A few years later Sabrazès and Muratet (1900) described " corpuscles mobiles " within the erythrocytes of *Hippocampus*. They were irregularly distributed, with usually five to ten granules within a cell.

In the same year these investigators extended their observations to several more fishes. In *Torpedo oculata* the granules were numerous and distributed diffusely around the nucleus. The cells usually contained as many as forty granules although some had as few as three, four, and five. All granules exhibited Brownian movement. They were unequal in size; some were coupled and some elongated or compressed. In *Raia pastinaca* the bodies were frequently oval and relatively large but were not so numerous as in *Torpedo*. The blood cells of *Syngnathus typhle* apparently did not contain any granules. In *Petromizon marinus* and *Alosa finta* the granules were present but not numerous. In adult *Anguilla vulgaris* they were absent. In 1902, however, Sabrazès and Muratet found opportunity to examine the blood of some young eels, 6 to 7 cm. long. In these, vital granules with limited Brownian movement were present in about twenty per cent of the erythrocytes. They varied greatly in size, with usually one to three present in any one cell. In *Torpedo marmorata* (Sabrazès et Muratet, 1908) the number and distribution of vital granules is very similar to *Torpedo oculata*.

Jokl (1925) made an intensive study of the erythrocytes of *Raia clavata* and *Raia batis* and found in the main that the red blood cells were very like those of *Torpedo* in the number, size, and distribution of the vital granules. Further observations on the elasmobranchs were made by Lewis and Lewis (1926), who described numerous diffusely scattered perinuclear bodies in the dogfish and skate (*Raia erinacea*). These workers also described one to three neutral red granules in the erythrocytes of the sculpin, but illustrated the erythrocytes of the hake and cunner as lacking any granular inclusions. Stolz (1928) found that the erythrocytes of *Cyprinus carpio* contained numerous granules diffusely distributed about the nucleus. Yoffey (1929) confirmed the observations of Jokl (1925) on the red blood cells of *Raia clavata* and *Raia batis*, and noted numerous fine basophilic granules in the mature cells of *Trigla gurnardus*. Dornesco and Steopoe (1930*a*) found that the erythrocytes of the dogfish are typical of the elasmobranchs in possessing numerous, scattered, actively motile granules. They (1930*b*) also investigated the blood of several marine teleosts, *Syngnathus acus, Blennius pholis, Solea vulgaris, Pleuronectes platessa, Gobius paganellus, Cottus bubalis, Labrus melops, Onos mustella* and *Nerophis lumbriciformis*. In all of these they found that the erythrocytes contained uniformly one granule, usually located eccentrically near one pole of the nucleus. The conditions in the erythrocytes of *Ameiurus nebulosus* are the same as in the marine teleosts (Dawson, 1931).

In the erythrocytes of the fishes studied the distribution of vital granules is not so variable as in the Amphibia, being limited chiefly to two types, single unipolar and multiple perinuclear, restricted or diffuse. The characteristic unipolar and bipolar clusters appearing in the red blood cells of many urodeles are not found. It has seemed advisable to extend the observations on the blood of fishes, with a view to obtaining more information regarding the relationships between the preëxistent and induced granules and the reticular substance as revealed by vital dyes.

Material and Methods

During the summer of 1931 the blood of seventeen different species of fish, taken in the vicinity of Woods Hole, was examined. Supravital staining was carried out by the dry dye-film method, using neutral red alone, neutral red in combination with Janus green, and brilliant cresyl blue. The concentration of neutral red, 1 : 1250, previously used on amphibian blood proved satisfactory in the case of fishes, but more Janus green had to be added to obtain a clear view of the chondriosomes. Films of brilliant cresyl blue were made with a saturated solution in absolute alcohol. Permanent preparations of supravitally stained blood were made by the method of Scott (1928). Smears stained by Wright's method were also prepared. In all cases the blood was freshly drawn from the heart of living fish.

It is essential that neutral red alone be used to check neutral red-Janus green B preparations, since the toxicity of Janus green may cause injuries which result in more rapid induction of bodies stainable with neutral red. On the other hand, Janus green B cannot safely be used alone, since bodies regularly stained with neutral red may take up Janus green and confuse the chondriosome picture. However, when the dyes are used in combination, the neutral red displays a greater affinity for the bodies which are regularly stained by it and the Janus green reaction is accordingly confined entirely to the chondriosomes. The brilliant cresyl blue was applied at such high concentrations that the nuclei were stained and the reticular patterns produced almost immediately, and there did not appear to be any progressive induction of formed bodies (granules) while the preparations were being studied for reticulation patterns.

Description

Before proceeding to the description of the reactions of the erythrocytes of the different fishes to vital dyes, certain general features of these reactions will be discussed. In most fishes there are sufficient immature red cells, in varying stages of differentiation, that the pictures presented after supravital staining lack uniformity. This makes the problem of interpretation more difficult, since the differences in hemoglobin concentration in some cases are scarcely perceptible. Differences in the degree of maturity of such cells are most strikingly demonstrated with brilliant cresyl blue and the reticulation patterns furnish an excellent index of cell age. The presence of young red cells in the blood is readily confirmed in smears differentially stained by Wright's method, where varying degrees of basophilia and polychromasia are clearly demonstrated.

As the erythrocytes mature the amount of material reacting with brilliant cresyl blue gradually decreases and the patterns of reticulation also undergo changes in form and distribution. In very young cells the entire cytoplasm is filled with fine, densely massed granules with little evidence of real reticulation. In succeeding stages of differentiation the pattern assumes a true reticular form. At first the meshes are small and distributed throughout the whole cell. Later the meshes are more open and the reticulation does not extend completely to the periphery of the cell, assuming the form of a perinuclear wreath. As the cells grow older, the meshes are still wider and the filaments eventually are partially interrupted, forming an open, fragmented wreath. As the reticular substance is further reduced, the filaments of the net are more frequently interrupted and the wreath-like form as well as the reticular appearance is lost. The more or less separated filaments then appear radially arranged about the nucleus, with very fine fragments interspersed between them. With the further disappearance of reticular substance the pattern is reduced to scattered, irregular, short filaments and fine, dust-like granules. In final stages the filamentous form may practically disappear, leaving a variable number of fine granules.

It is difficult to determine whether the reticular substance, which is demonstrated with concentrations of the vital dye sufficiently high to stain the nuclei immediately, is of the same constitution as the relatively large granules which may be induced in similar blood cells by a prolonged exposure to the same dye in a lesser concentration, which will not stain the nucleus or produce reticulation. At present, it seems impossible to decide this question. On the other hand, it is frequently possible to distinguish clearly between preëxistent vital granules and the induction patterns, since in many cases the former may be seen in fresh untreated cells or may be demonstrated in fixed cells by a variety of reliable technical methods.

With high concentrations of brilliant cresyl blue, nuclei, nucleoli (when present), preëxistent and induced granules, and reticular substance are all stained. The nuclei are pale blue, nucleoli deep blue, all granules deep blue-purple and the reticular substance a red-violet. The nucleoli, varying in number from one to three, are large and conspicuous in the erythroblasts but become progressively smaller as the cells mature. They may be distinguished as minute bodies in cells of the "wreath" stage but seldom persist in more mature forms. The difference in the color reaction of the granules and the reticular substance may or may not be significant. The granules are sharply limited, dense, and highly refractile, while the reticular substance has an irregular appearance as if formed by the aggregation of very fine particles. The difference in physical state accordingly may explain the different shades presented by the two types of bodies.

The form and distribution of chondriosomes within the erythrocytes of the fishes vary in the different stages of maturity, but are nevertheless quite characteristic for the type of cell. In the younger stages they are quite numerous, granular, and diffusely scattered throughout the cytoplasm. In later stages the number is greatly reduced and they are more closely aggregated about the nucleus. Their form, too, may be changed, many appearing as long, tortuous bodies. In fully mature cells the chondriosomes are usually entirely of the filamentous type, although occasional granular forms are seen. Some of these are not true granules but represent filaments oriented lengthwise between the two flattened surfaces of the erythrocyte and may be observed to shift in position as the cell is modified by the injurious effects of the Janus green.

It is difficult to obtain sharp images of chondriosomes in the fresh cell, and relatively high concentrations of dye must be used. As the staining progresses the chondriosomes are first seen as hazy blue-green bodies, and at this time the nuclear outline is also indistinct. Shortly both the nuclear membrane and chondriosomes are distinctly seen and the cytoplasm of the cell appears clearer. While these changes are taking place striking movements of the mitochondria may be observed. Filaments which were closely applied to the nucleus may frequently swing out to lie at right angles to the nuclear surface or may move completely away, and others which were seen on end may assume a position parallel with the flattened surface of the cell.

For purposes of description the fishes studied at Woods Hole will be divided into two major groups, based on the distribution of the primary or preëxistent granules as demonstrated by low concentrations of neutral red. In the first group the vitally stained granules are usually single, with occasionally one or more accessory bodies, and are located eccentrically near one pole of the nucleus. In the second group the granules are numerous and may either have a definite perinuclear arrangement or be scattered more or less diffusely throughout the cytoplasm. The reaction of the primary granules to Wright's stain might also be used as a basis of classification, since in some fishes the granules uniformly give a basophilic reaction; in others this reaction is limited to a varying number of cells; while in still others the granules always remain unstained. The three second groups would form natural subdivisions of the major Group I, which is based primarily on the distribution of granules, but in Group II this would not hold true since in no instances do the perinuclear or diffusely arranged granules react uniformly with Wright's stain.

Group I

Toadfish, *Opsanus tau* (Linnæus). With neutral red the primary granules ordinarily appear as single, unipolar bodies, although one or more accessory granules are often encountered (Fig. 1, *b* and *c*). The accessory granules are usually small, but in some cases all the granules are of equal size, resembling the variations described for *Ameiurus* (Dawson, 1931). After long exposure to neutral red the granules are increased in number and tend to form clusters about the primary bodies. Later, other secondary granules appear irregularly throughout the cell.

The mitochondria are relatively few, varying in number from three to six. They usually appear as wavy filaments but are frequently dilated to encapsulate a large spherical refractile body. These bodies are readily seen after the Janus green has been reduced. They do not take up neutral red and their significance is not known.

Very little reticular substance is demonstrated by brilliant cresyl blue. The filaments are short and are usually radially arranged about the nucleus. Some end in contact with the cell membrane. Primary granules and a variable number of secondary granules are stained with this dye. In the cells with the least reticulation practically no induction of granules has taken place (Fig. 1, *d*). The cells with the most reticulation contain many more granules (Fig. 1, *a*). All the cells appear relatively mature. An occasional cell in the "wreath" stage with a clear border was encountered, but there were no younger stages.

In smears stained by Wright's method the primary granules appear as distinct basophilic bodies. No basophilic erythrocytes are present and only a few cells exhibit polychromasia.

Tautog, *Tautoga onitis* (Linnæus). The neutral red patterns are quite similar to those of the toadfish (Fig. 4, *a* and *b*). A number of erythrocytes possess granular mitochondria, but in the majority of the cells they are filamentous. The reticular substance is scanty in most cells (Fig. 4, *d*), but a few "wreath" stages were seen (Fig. 4, *c*). Occasional small cells (erythroblasts) with a dense reticulation and a conspicuous nucleolus are present.

With Wright's stain the primary granules are stained blue and a few erythrocytes show basophilia and polychromasia. The number of immature red cells is, however, very small.

Cunner, *Tautogolabrus adspersus* (Walbaum). The erythrocytes of this fish very closely resemble in their staining reactions those of the two preceding forms (Fig. 6, *a* and *b*). Fewer immature cells were noted than in the tautog, and in general the reticulation patterns are very meager (Fig. 6, *c* and *d*). The primary granules also appear as basophilic bodies with Wright's stain.

Sea Bass, *Centropristes striatus* (Linnæus). The primary neutral red bodies are the same as in the erythrocytes of the preceding fishes (Fig. 3, *a*). Induction of secondary granules occurs rather freely (Fig. 3, *b*), but even when the secondary staining effect has appeared there are a number of apparently mature cells which do not show any reaction to the dye. The mitochondria are relatively few, the filamentous form predominating.

With brilliant cresyl blue many cells fail to show any reticular patterns but contain only granules such as are seen after moderate induction with neutral red. Other cells contain a variable number of reticular filaments (Fig. 3, *c*), but the "wreath" stage was observed in only a few cells (Fig. 3, *d*). Occasional young cells with a fine, dense reticulum and conspicuous nucleoli were encountered.

In the sea bass virtually all the erythrocytes stain orthochromatically with the eosin in Wright's stain. Polychromasia is rarely seen and occasional round basophilic erythroblasts are present. The primary granules demonstrated so readily with neutral red are not usually stained in the smears. Some of the larger granules appear as distinct basophilic bodies, other smaller granules are barely distinguishable, and many cells appear not to have any granular inclusions.

Sea Robin, *Prionotus carolinus* (Linnæus). The erythrocytes of the sea robin in their staining reactions are very like those of the sea bass (Fig. 2). The mitochondria, however, usually appear as short rods and granules rather than filaments. The reticular patterns are very sparse, being represented by scattered particles radially aligned about the nucleus (Fig. 2, *d*). All the cells appear to be mature. This conclusion is supported by the staining reactions of the erythrocytes with Wright's stain. No granular inclusions could be distinguished in the stained smears.

Scup, *Stenotomus chrysops* (Linnæus). The neutral red patterns and mitochondria present no unusual features (Fig. 7, *a* and *c*). The reticular patterns are quite variable, although in the majority of cells the filaments are reduced to a minimum (Fig. 7, *b*). Young cells with a dense reticulum and conspicuous nucleoli are frequently seen and stages with fragmented open-meshed "wreaths" are quite numerous (Fig. 7, *d*). The presence of such immature cells is confirmed by an examination of stained smears, but the preëxistent or primary granules were not demonstrable as basophilic bodies.

Butterfish, *Poronotus triacanthus* (Peck). The primary granule is characteristically present in neutral red preparations (Fig. 8, *b*) and induction of new granules proceeds very slowly. The chondriosomes are usually granular. No long filamentous forms were observed. The reticular patterns and numbers of immature cells are about the same as in the scup (Fig. 8, *a*, *c*, and *d*).

Variegated minnow, *Cyprinodon variegatus* Lacépède. The primary neutral red bodies usually appear as single or double bodies and induction of secondary bodies occurs slowly. The mitochondria are predominantly of the granular type and are relatively numerous (Fig. 11, *a* and *b*). In the majority of the cells the reticular material is very scanty (Fig. 11, *c* and *d*), but in a few cells complete perinuclear "wreaths" of reticular material were seen. Occasional younger cells, possessing large nucleoli and dense reticulations, were also noted. The primary neutral red bodies of mature cells did not give a basophilic reaction with Wright's stain, but in many polychromatic cells they could be distinctly seen as basophilic granules.

Mummichogs, *Fundulus heteroclitus* (Linnæus) and *F. majalis* (Walbaum). The behavior of the erythrocytes is essentially alike in these two species. The primary neutral red bodies are characteristically present (Fig. 12, *b* and *c*). The reticular substance in general is scanty, appearing as radially arranged filaments (Fig. 12, *d*). A few cells in the "wreath" stage and an occasional young cell with a large nucleolus were seen. The primary granules were not demonstrated by Wright's method.

Common eel, *Anguilla rostrata* (Le Sueur). The neutral red bodies are very minute, almost at the limit of visibility (Fig. 5, *a* and *d*). However, they gradually increase in size on exposure to the dye and secondary granules slowly appear (Fig. 5, *b*). The mitochondria are few in number and chiefly of the long, sinuous type. Brilliant cresyl blue reveals the presence of large numbers of immature erythrocytes, the reticular substance varying in distribution from complete "wreaths" to scattered isolated filaments and granules (Fig. 5, *c*). In stained smears many erythrocytes show varying degrees of cytoplasmic basophilia. The primary granules frequently appear as basophilic bodies in cells which stain orthochromatically in eosin or show a slight polychromasia. However, they were not distinguishable in more basophilic cells.

Sand dab, *Hippoglossoides platessoides* (Fabricius). The primary vital granules are readily demonstrated as single or double granules (Fig. 9, *a*). With longer exposure to the dye secondary granules, usually grouped in clusters, quickly appear (Fig. 9, *c* and *d*). Chondriosomes of both filamentous and granular types are present. Practically all the cells are mature, showing a very sparse reticulation (Fig. 9, *b*). An occasional cell with an incomplete open-meshed "wreath" was seen. A few basophilic erythrocytes were demonstrated with Wright's stain. The primary granules were frequently seen as basophilic bodies in erythrocytes which exhibit a slight polychromasia but could not be distinguished in mature cells.

Group II

In the erythrocytes of the fishes of this group the primary granules are not limited to a single or double unipolar body but are relatively numerous and have a perinuclear distribution.

Menhaden, *Brevoortia tyrannus* (Latrobe). In the menhaden the granules are very small, frequently barely visible at a magnification of nine hundred diameters. The granules are either arranged in a single definite line about the nucleus or scattered somewhat irregularly throughout the cytoplasm (Fig. 10, *a* and *b*). The irregular distribution of granules appears to be characteristic of the oldest cells. On longer staining with neutral red they increase in both size and number. Mitochondria of both the filamentous and granular types are present in restricted numbers.

All stages of maturating erythrocytes were encountered, but the majority of the cells were fully differentiated. The reticular patterns vary all the way from a dense compact granular mass filling the entire cell to the mature condition in which only fine particles and scattered, short filaments are present (Fig. 10, *c* and *d*). In stained smears erythrocytes in the different stages of differentiation are clearly shown and the numerous perinuclear primary granules are seen distinctly as basophilic bodies in both polychromatic and mature cells.

Alewife, *Pomolobus pseudoharengus* (Wilson). The erythrocytes of the alewife are essentially like those of the menhaden in all their reactions to the dyes, but more immature cells are present (Fig. 14). Many of the younger cells contain large, clear, refractile globules which appeared as vacuoles in stained smears.

Mackerel, *Scomber scombrus* Linnæus. The erythrocytes of the mackerel differ only slightly from those of the menhaden and alewife (Fig. 13). The primary neutral red patterns are alike. Mitochondria of the filamentous type predominate. More immature cells are present than in the alewife and the reticulation patterns are accordingly very variable. The red cells react rapidly with neutral red and many secondary granules may develop. The primary neutral red granules are demonstrated as basophilic bodies by Wright's stain, but much more care must be taken in the differentiation of the stain.

Smooth dogfish, *Mustelus canis* (Mitchill) and spotted skate, *Raja diaphanes* Mitchill. The vital staining reactions of the erythrocytes of both these forms have been described by several investigators. The granules appearing after exposure to neutral red are very large and numerous (Figs. 15 and 17). The mitochondria are usually long and filamentous. Induction of new granules occurs rapidly. There is a relatively high proportion of immature red cells, and the reticulation patterns are very variable. In old cells the reticular substance is greatly reduced, appearing as scattered particles and short filaments.

With brilliant cresyl blue both vital granules and reticulation patterns are demonstrated as in the teleosts, but on long standing the dye in the granules disappears while the reticular substance remains brilliantly stained. The reduction of the dye in the granules appears to be characteristic only of the elasmobranchs and has been previously reported by Jokl (1925). Although the granules are large and readily seen in fresh unstained preparations, they cannot be demonstrated in smears stained by Wright's method and do not give a basophilic reaction.

THE RELATIVE DEGREE OF DIFFERENTIATION OF THE MATURE ERYTHROCYTES OF VERTEBRATES

In the running description of the findings in the erythrocytes of the different species of fish, the staining reactions described related particularly to the predominating cells, presumably the mature ones. During the course of the study it became increasingly obvious that no granules, either preëxistent or induced, were present in young cells exhibiting a complete, dense reticulation pattern. In somewhat older stages the primary granules were readily demonstrated, but induction of new granules either did not occur or proceeded very slowly. This secondary reaction varied greatly in different species, but the general trend was the same in all. In more mature stages induction usually occurred more readily, but in some old, perhaps senile, cells, which gave practically no reticulation reaction with brilliant cresyl blue, the secondary response to vital dyes again decreased.

The terms " primary " or " preëxistent," and " secondary " or " induced " are used here without reservation to designate the granules which are under discussion, since the cumulative evidence gathered from studies on fishes and amphibians indicates that such a distinction is valid. The granules which stain so readily with neutral red are frequently seen as refractile bodies in fresh preparations, and in dry-fixed smears are frequently demonstrated as basophilic inclusions. In most cases the characteristic form and location of these elements make their identification easy when they are rendered visible by other than supravital methods. Dornesco and Steopoe (1930*a*, 1930*b*) have also successfully blackened these primary bodies with silver nitrate methods (Da Fano and Cajal) in both teleosts and elasmobranchs. Their demonstration by a silver method in the elasmobranch erythrocyte is significant, since in these cells the granules fail to give a basophilic reaction in carefully stained smears.

The reality of the secondary granules cannot be disputed since they can be observed as they arise within the cells. Accordingly we have to deal with three distinct morphological entities, at least as they are demonstrated by supravital methods. The question of their chemical constitution and relationships is a baffling one and apparently little further progress can be made in this regard until better methods of study are devised.

Nevertheless, the characteristic reactions to vital dyes may be legitimately used as evidences of the progressive changes which occur within the differentiating erythrocyte, and such reactions therefore constitute useful criteria for determining the degree of differentiation reached by the mature erythrocytes of a given species. The criteria of maturity in one species are not necessarily valid in every detail for erythrocytes of another species, since the pictures obtained by vital dyes may vary in different groups of animals. Maturity of erythrocytes in general can best be defined as the stage at which the cell acquires its maximum concentration of hemoglobin, and in normal animals cells of this type should predominate in the circulation. The degree of concentration of hemoglobin in mature cells in a given species is relatively constant and is closely correlated with the acquisition of characteristic staining reactions, but similarity of staining patterns in different species does not by any means indicate that the different erythrocytes have acquired the same concentration of hemoglobin. In other words, the erythrocytes of the different vertebrates are mature at varying levels of differentiation, the latter being measured by such staining reactions as are usually regarded as evidences of immaturity in the most highly differentiated red cells.

During the differentiation of the vertebrate erythroblast a striking series of changes occurs. Some are readily demonstrated in fixed preparations while others are adequately revealed only by supravital staining. Most of these changes are common to the erythrocytes of all vertebrates, but in the mammals an extreme degree of specialization is encountered. During differentiation the nuclear-cytoplasmic ratio changes greatly, the nucleoli undergo a gradual involution and may disappear, the distribution of chromatin in the nucleus is modified, and the basophilia of the cytoplasm is gradually lost and replaced by the eosinophilia of the hemoglobin. Also the volume of mitochondrial substance is progressively reduced, and frequently the form of the individual elements is changed.

The gradual reduction of the reticular substance, definitely correlated with a decreasing basophilia of the cytoplasm, is the most striking feature of the maturing erythrocytes when seen in supravital preparations. Less conspicuous changes involve the appearance and behavior of both types of granular inclusions, preëxistent and induced. Primary granules are usually absent from very young cells and disappear in a later but somewhat variable stage of differentiation. When they first appear they do not give a basophilic reaction in stained smears, but later in many instances they are characteristically basophilic. In mature cells of some animals the basophilic reaction is subsequently lost and the bodies are again demonstrated only by supravital methods. Moreover, in some vertebrates the primary granules may entirely disappear while in others they persist and assume characteristic patterns of distribution. Other granules of similar morphology and behavior may appear in cells that have stood in preparations for some time. Apparently induction of granules can occur only after some degree of differentiation of the erythrocyte has been attained, but the ability to respond in this manner may persist until full maturity is reached, and in many instances induced granules may appear even after the primary granules are no longer in evidence. In the mammals, however, the nucleus and all the cytoplasmic inclusions eventually disappear completely.

In the different vertebrates each of these changes in the maturing erythrocyte may take place at a different rate and to a varying degree. Accordingly, with the exception of the mammals, it is not always easy to decide which cells represent at maturity the more fully differentiated stage. In all vertebrates below the mammals the cells are characteristically nucleated with a few striking exceptions in the Amphibia (Emmel, 1924). In all of these animals the basophilia is eventually replaced by eosinophilia. The nucleoli may disappear; the nuclear-cytoplasmic ratio undergoes considerable changes, the nucleus becoming condensed and acquiring a characteristic chromatic pattern. That is, in ordinary stained smears the mature nucleated erythrocytes of vertebrates appear essentially alike except that they vary greatly in size. However, with the more delicate methods of supravital staining, quite striking differences are brought out. Accordingly, the patterns of granulation and reticulation afford the best criteria of the degree of differentiation of these cells, and of these two criteria the degree of persistent reticulation is probably the better since the reticular material is continuously present in the cell while the granules have a variable and much more complicated history. Still, the latter criterion cannot be entirely disregarded in comparing the nucleated red cells of the vertebrates.

Adequate data for the comparison of the erythrocytes of vertebrates are available for fishes and amphibians, but the blood of reptiles and birds has not been studied so extensively by means of vital dyes. In order to secure first-hand information concerning the conditions in the reptiles and birds, supravital preparations of blood from the painted turtle, horned toad, fence lizard (*Sceloporus undulatus*), and the common fowl were studied.

In the fishes (both elasmobranchs and teleosts) and birds little reticular substance is present in the mature cells (Fig. 18, *a*, *c*, and *d*), being represented mostly by scattered filaments and granular fragments. My observations on the fowl differ from those of Doan, Cunningham, and Sabin (1925), who report that the final stages of reticulation consist in a few discrete bodies stainable with vital dyes. These bodies are readily demonstrated by low concentrations of either neutral red or brilliant cresyl blue (Fig. 18, *b*), but when brilliant cresyl blue in sufficiently high concentration to stain the nuclear reticulum is used, scattered fragments and short filaments are also uniformly present in the cytoplasm. Brilliant cresyl blue is apparently reduced to some extent in the cell and the demonstration of persisting fragments of reticulum is possible only when the dye is present in considerable excess. In the amphibians and reptiles, on the other hand, a definite, more or less complete reticular pattern can be demonstrated in the mature erythrocytes, but in most instances the amphibian erythrocytes contain the greater amount of reticular substance (Figs. 16, 19, 20, and 22). If the degree of persistence of reticulation is regarded as evidence of the degree of differentiation attained by the mature erythrocyte, it must be concluded that the red blood cells of fishes and birds are relatively more highly differentiated than those of the amphibians and reptiles.

The amount of material demonstrable as so-called primary vital granules in the mature erythrocytes of the several classes of vertebrates cannot be readily correlated with the degree of persistent reticular substance, but the history of the vital granules indicates that their presence in red blood cells is in some degree a measure of relative maturity. It has been already pointed out that the reticular substance is at a maximum in the young cells and gradually decreases as the erythrocytes mature, while in general the granular substance is not present at all until the cells are partially mature.

It is practically impossible to make any significant generalizations regarding the vital granules. The erythrocytes of each species must be considered separately if any accurate conclusions are to be drawn regarding relative maturity of the cells. It has been held by many that the granular material is but a phase of the filamentous reticulum. In very young cells the reticular substance is definitely granular before acquiring the filamentous form. The granular form is doubtless dependent on its concentration within the cell and is the result of the agglutinating or precipitating effect of the vital dye. The primary granules are, however, definite, discrete bodies, frequently distinguishable in fresh unstained cells, and may often be demonstrated in fixed material. They are apparently associated in some way with cell metabolism, and when the erythrocytes are exposed to a penetrating dye it accumulates first of all in these preformed structures. Whether the secondary or induced bodies are derived from reticular material is less easy to determine. But certain lines of evidence would appear to indicate that they are the result of a specific reaction of the cytoplasm to an excess of dye and do not represent local, sharply delimited accumulations of reticular material (Chlopin, 1927).

The history of the behavior of these three elements in the maturing erythrocytes further suggests that they are separate entities. In elasmobranchs the primary granules of mature cells are large and numerous when the reticular substance is reduced to the same degree as in the teleosts. In the amphibians the history is also variable, granules being either present or absent, depending on the species (Arrigoni, 1908; Beams, 1930; Dawson and Charipper, 1929; DeRoo and Ufford, 1930; Goda, 1929; Hibbard, 1928; Jordan, 1925; Stolz, 1928), while the reticular material persists as a fairly complete, open network. In *Triturus viridescens* the erythrocytes rarely contain any granules either primary or secondary, but as Nigrelli (1929) has shown, the reticulation in mature cells is relatively abundant (Fig. 16). Other similar examples could be cited.

In the reptiles the reticulation is quite abundant in mature cells but the primary granules are single in the alligator, horned toad, and fence lizard, and clustered at one pole in the painted turtle. The granules in these several species can also be demonstrated as basophilic bodies in dried smears stained by Wright's method (author's observations), and the question of their being induced bodies cannot be raised. In the common fowl and pigeon, Doan, Cunningham, and Sabin (1925) regard the single vital granule as a vestige of the reticular substance. Forkner (1929) also states that in the domestic fowl the cytoplasm of the erythrocytes contains, after staining with neutral red, from none to several small, reddish brown bodies which are usually near the nucleus but often move about and may be far out near the cell border. These bodies are not demonstrated in smears stained by Wright's method but, as pointed out earlier, they have probably been demonstrated by other methods and mistaken for centrosomes. Their close resemblance to similar structures in teleosts and reptiles which can be shown to be primary bodies lends strength to the view that they are preëxistent in the fowl erythrocytes, and are not produced by a reaction of the dye with remnants of the reticular substance. In view of the evidence accumulated from a study of the nucleated erythrocytes of vertebrates, the author is inclined to accept the conclusion of Michels (1931) that the reticular substance in reticulocytes has no genetic relation to the vital granules of the mature red cells, but would disagree with his acceptance of the view that vital granules are surface structures, either precipitates of the stain or stained precipitates of the plasma. After a study of the irregular behavior of the vital granules in nucleated erythrocytes, it does not seem surprising that such granular cytoplasmic inclusions do persist even after the nucleus, chondriosomes, and reticular substance have disappeared from the mammalian cell. The appearance of primary granules in the cytoplasm is apparently a constant phenomenon in the maturation of the erythrocytes. It is only in the mammalian erythroplastid that they uniformly completely disappear and in this instance they mark the acme of erythrocyte differentiation. They, however, are not always the last vestige of immaturity to disappear, since in some higher urodeles and several anurans they disappear while the reticular substance is still present in relatively large amounts.

The appearance of secondary or induced granules in erythrocytes following exposure to vital dyes apparently has only a very limited relation to the degree of differentiation attained by such cells. It is true that young cells with a high concentration of reticular substance react slowly and to a very limited degree to dyes, but the amount of reaction obtained in more mature cells is also very variable and seems not to be directly determined by the degree of differentiation attained. Rather the reaction appears to be species specific and to depend on the permeability of the cell and other factors inherent in the cytoplasm of the given species. Also the degree of the reaction with the cytoplasm cannot be correlated with the presence of a certain amount of reticular substance, since as much induction may occur in mature erythrocytes of fishes with a minimum of reticulation as in those of amphibians and reptiles where the reticular substance persists in greater amounts. The induction phenomenon in cells containing primary granules is usually confined at first to the areas of the cell containing such preëxistent bodies. In the early stages of induction the primary granules themselves become enlarged and new granules then appear in their immediate vicinity. Later new bodies may form irregularly throughout the cytoplasm.

The phenomenon of induction in erythrocytes appears to be closely related to the " krinome " reaction to vital dyes described by Chlopin (1927) for many other cells of the animal body. Freely penetrating stains such as the basic dyes appear to accumulate within the cytoplasm of the red blood cells and appear first in the preformed bodies when they are present. Later, as more dye is accumulated, it is segregated by some reaction of the cytoplasm into newly formed structures. In some erythrocytes this accumulation and subsequent segregation of dye within the cytoplasm appears to continue progressively and to surprising limits, while in other erythrocytes the reaction proceeds only to a minimum extent. As has been already noted, many of the external factors influencing this reaction are known, but the factors within the cytoplasm, which are apparently of utmost importance, are unknown. The reaction, as in the anurans (Beams, 1930), proceeds as well in mature cells without preformed bodies as in cells in which preformed bodies are numerous and conspicuous. The final loss of the ability to react, as in the most highly differentiated mammalian erythrocytes, would seem to indicate that the degree of differentiation attained by the cell in some way determined or limited the cytoplasmic reaction to the dye. Such a conclusion, however, is rendered more or less untenable by the irregular behavior of the nucleated erythrocytes of other vertebrates, whose degree of differentiation at maturity can be estimated by the degree of persistence of reticulation. In such cells, except in very young stages, there is no significant correlation between the relative degree of differentiation attained and the degree of neo-formation of vitally stained bodies.

Before concluding this discussion of the relative degree of differentiation of the mature vertebrate erythrocytes, one other morphological feature of maturing erythrocytes should be mentioned, although the observations on it are not all comprehensive. It has already been noted that with relatively high concentrations of brilliant cresyl blue the nucleoli in immature cells of the fishes appeared as deep blue bodies in a light blue, apparently homogeneous nucleus, the nuclear reticulum not being shown with such concentrations of the dye. The nucleoli (plasmosomes) are relatively large in young cells and vary in number, but there are seldom more than three in any cell. They grow smaller as the erythroblasts differentiate and usually appear as single, small, spherical bodies. After the stage at which the reticular substance appears as an open-meshed, almost complete network the nucleoli are more rarely seen, and in the mature cells containing scattered fragments of reticulum they are usually absent. In the catfish, however, nucleoli are uniformly present in the mature erythrocytes.

Nucleoli are not readily demonstrated in the mature cells of the Amphibia by brilliant cresyl blue, but they may be stained if concentrations of dye sufficiently high to bring out the chromatin reticulum of the nuclei are used. The dye, however, must not be intense enough to stain the chromatin a dark blue or the nucleoli are obscured (Fig. 16). In such preparations of the erythrocytes of *Necturus* and *Triturus* the nucleoli, numbering from one to four, appear as dark blue bodies lying between the coarse bars of light blue chromatin. They are somewhat irregular in form and are not so sharply outlined as in the fishes. At first it seemed doubtful if these bodies were nucleoli, but a comparison of the mature cells with younger stages in the circulation appears to establish their plasmosomal nature.

In the reptiles studied (horned toad, fence lizard, and painted turtle) for this feature of the mature erythrocyte, the nucleolus is uniformly found as a single spherical body in all mature erythrocytes, and is a striking feature of all properly stained preparations (Figs. 19, 20, and 22). Immature cells were rarely encountered in the blood of these animals and no comparisons with the nucleoli of younger erythrocytes were made. In the blood of the fowl no nucleoli could be distinguished in mature cells; but in an occasional immature cell, still in the stage with a more or less complete reticular net, small single spherical nucleoli were observed.

This method of demonstrating nucleoli, supravitally with brilliant cresyl blue, in red blood cells does not appear to have been previously employed. It seems to be a delicate method and to give clear pictures of nucleoli even when they cannot be easily demonstrated in fixed and stained preparations. In many amphibian erythrocytes brilliant cresyl blue also stains the achromatic contents of the nucleus a reddish violet, while the chromatin is a light blue and the nucleoli deep blue. In such cells the nucleoli appeared to be imbedded in the achromatic substance.

These observations on the persistence of the nucleoli are correlated in a very satisfactory manner with the findings on the degree of persistence of reticular material and afford additional evidence that the mature erythrocytes of fishes and birds are relatively more highly differentiated than those of amphibians and reptiles.

The degree of differentiation of erythrocytes of the several classes of vertebrates as determined by the criteria of persistent reticulation and presence of nucleoli also seems to correlate fairly well with the size of these cells. The Amphibia as a class have the largest erythrocytes (Fig. 21). Reptiles have blood cells next in size and fishes come next, then birds and mammals. It is difficult, however, to conceive that cell size could directly influence the degree of persistent reticulation or the persistence of nucleoli. The presence of primary granules might possibly be dependent to some extent on this factor, since the products of metabolism might be less readily eliminated from larger cells and temporary accumulations be segregated in the cytoplasm in the form of granules.

Summary

The reactions of the mature erythrocytes of seventeen species of fishes to the vital dyes neutral red, Janus green B, and brilliant cresyl blue, have been studied. In most teleosts the primary vital granules are readily demonstrated by neutral red and consist of one or two small granules eccentrically placed near one pole of the nucleus, but in the menhaden, alewife, and mackerel the primary granules are most numerous and are either arranged in a single definite line about the nucleus or scattered irregularly throughout the cytoplasm. In the elasmobranchs the granules are large, numerous, and scattered. In a majority of the teleosts the primary granules may be demonstrated as basophilic bodies in dry films stained by Wright's method and are also frequently seen in fresh unstained preparations.

Secondary or induced granules may also appear in the cytoplasm of cells exposed for long periods to the dye. The degree of induction of new bodies does not appear to depend entirely on external factors but is determined to a large extent by factors inherent in the cytoplasm of the given species. In general the mitochondria are filamentous, reduced in number, and lie in close contact with the surface of the nucleus.

The reticular substance in all mature erythrocytes of the fishes is greatly reduced and appears either as short irregular filaments or as minute granular remnants. It is best demonstrated with brilliant cresyl blue.

An attempt is made to compare the relative degree of differentiation attained by the mature erythrocytes of the several classes of vertebrates. The following criteria have been considered: changes in nuclear-cytoplasmic ratio; chromatin distribution in the nucleus; involution of the nucleoli; loss of basophilia; changes in the form, distribution, and volume of mitochondrial substance; reduction of reticular substance; amount of primary granules; and degree of induction of secondary granules. Of these criteria the degree of persistence of reticulation has been found to be the most consistent, and on this basis the several classes of vertebrates are arranged in the following ascending order of relative differentiation attained by their erythrocytes at maturity: amphibians, reptiles, fishes, birds, and mammals. This arrangement is also supported by the behavior of the nucleoli, which persist in the erythrocytes of amphibians and reptiles but are not usually demonstrated in the mature cells of fishes and birds.

The history of the primary and secondary granules is less regular and consequently less useful for measuring the relative differentiation attained by the cells of different classes of vertebrates. However, within a given class of vertebrates it is concluded that the presence of a large number of primary granules or the rapid induction of new granules in mature cells may be regarded as supplementary evidence of a lesser degree of differentiation. The presence of primary granules or the degree of induction of new granules, however, cannot always be correlated with the degree of persistence of reticulation.

It is also concluded on the basis of this survey of the vertebrate erythrocyte that primary granules, secondary granules, and patterns of reticulation as revealed by vital dyes, must be regarded as three separate entities which are not genetically related.

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EXPLANATION OF PLATES

All drawings are from dry preparations and were outlined at the same magnification by means of a camera lucida. The details were filled in free-hand from sketches of the fresh cells. Erythrocytes stained with brilliant cresyl blue to demonstrate patterns of reticulation are shown with solid nuclei. All others, with the exception of Fig. 21, were stained either with neutral red and Janus green B or neutral red alone. The mitochondria are usually filamentous but some granular forms are present. Ordinarily they may be distinguished by their juxta-nuclear position.

PLATE I

Explanation of Figures

1. Erythrocytes of the toadfish showing, (*a*) induction of granules and reticulation; (*b* and *c*) primary granules and mitochondria; (*d*) primary granules and reticulation.

2. Erythrocytes of the sea robin showing, (*a* and *b*) the primary granules and mitochondria; (*c*) induction of granules after twenty minutes; (*d*) reticulation.

3. Erythrocytes of the sea bass showing, (*a*) primary granules and mitochondria; (*b*) induction of granules after twenty minutes; (*c*) reticulation in a mature cell; (*d*) reticulation in an immature cell which possesses a nucleolus.

4. Erythrocytes of the tautog showing, (*a* and *b*) primary granules and mitochondria; (*c*) reticulation in an immature cell; (*d*) reticulation in a mature cell.

5. Erythrocytes of the eel showing, (*a* and *d*) primary granules and mitochondria; (*b*) induction of granules after twenty minutes; (*c*) reticulation in a mature cell.

6. Erythrocytes of the cunner showing, (*a* and *b*) primary granules and mitochondria; (*c* and *d*) reticulation in mature cells.

7. Erythrocytes of the scup showing, (*a* and *c*) primary granules and mitochondria; (*b*) reticulation in a mature cell; (*d*) reticulation in an immature cell.

8. Erythrocytes of the butterfish showing, (*a*) reticulation in a mature cell; (*b*) primary granules and mitochondria; (*c* and *d*) reticulation in immature cells.

9. Erythrocytes of the sand dab showing, (*a*) primary granules and mitochondria; (*b*) granules and reticulation; (*c* and *d*) induction of granules after twenty minutes.

10. Erythrocytes of the menhaden showing, (*a*) perinuclear primary granules and mitochondria; (*b*) granules after twenty minutes; (*c*) reticulation in a mature cell; (*d*) reticulation in an immature cell.

11. Erythrocytes of *Cyprinodon* showing, (*a* and *b*) primary granules and granular mitochondria; (*c* and *d*) reticulation in mature cells.

12. Erythrocytes of *Fundulus majalis* showing, (*a*) induction of granules after twenty minutes; (*b* and *c*) primary granules and mitochondria; (*d*) reticulation in a mature cell.

13. Erythrocytes of the mackerel showing, (*a*) reticulation in an immature cell; (*b*) perinuclear primary granules and mitochondria; (*c*) induction of granules after twenty minutes; (*d*) reticulation in a mature cell.

14. Erythrocytes of the alewife showing, (*a*) induction of granules after twenty minutes; (*b*) reticulation in a mature cell; (*c*) primary granules and mitochondria; (*d*) reticulation in an immature cell.

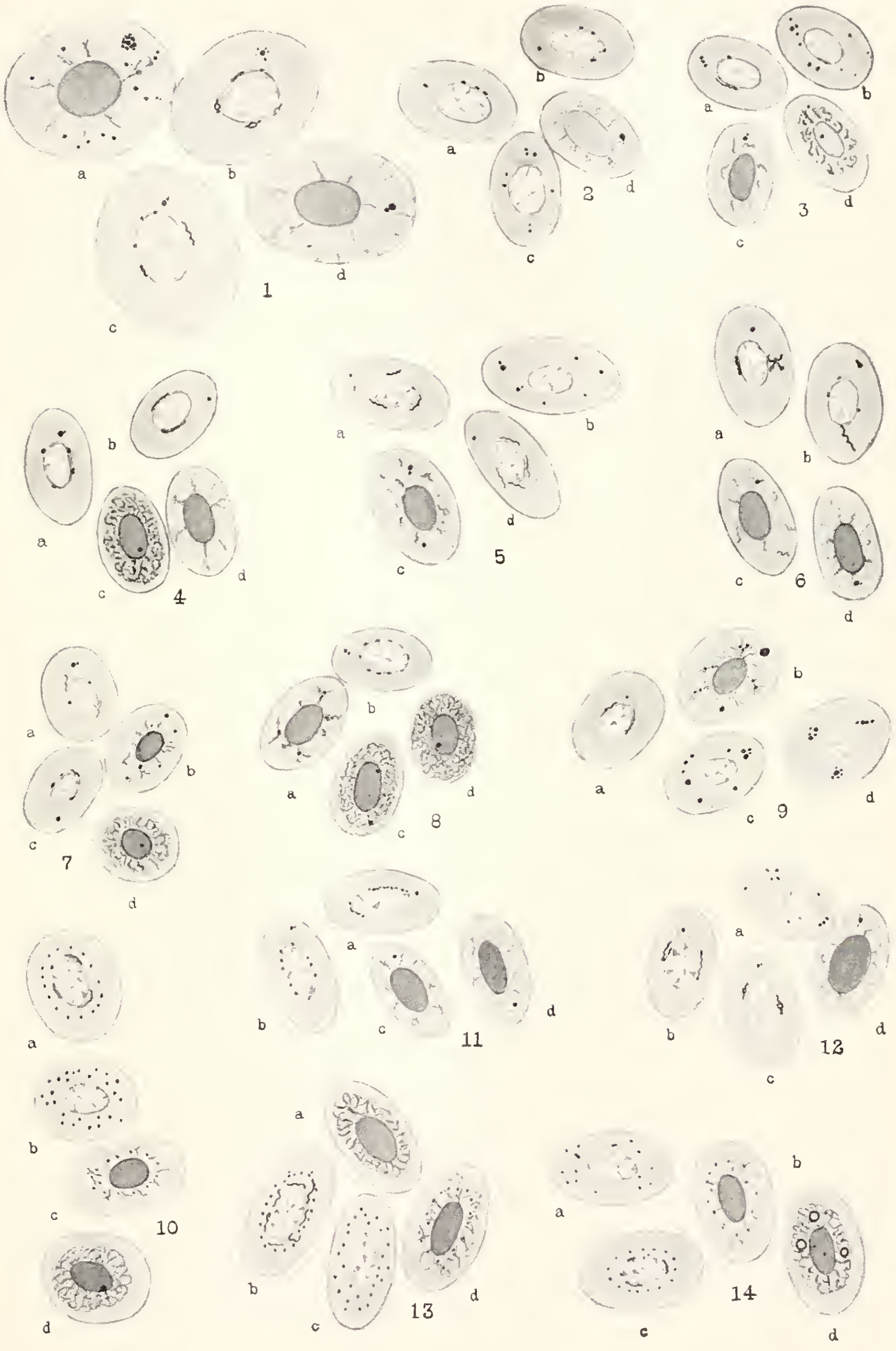
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PLATE II

Explanation of Figures

15. Erythrocytes of the smooth dogfish showing, (*a*) the primary granules and mitochondria; (*b*) reticulation in a mature cell; (*c*) reticulation in an immature cell.

16. An erythrocyte of *Triturus viridescens* showing reticulation of a mature cell. Note the complete absence of granules.

17. Erythrocytes of the spotted skate showing, (*a, b,* and *c*) primary granules and mitochondria; (*d*) reticulation in a mature cell.

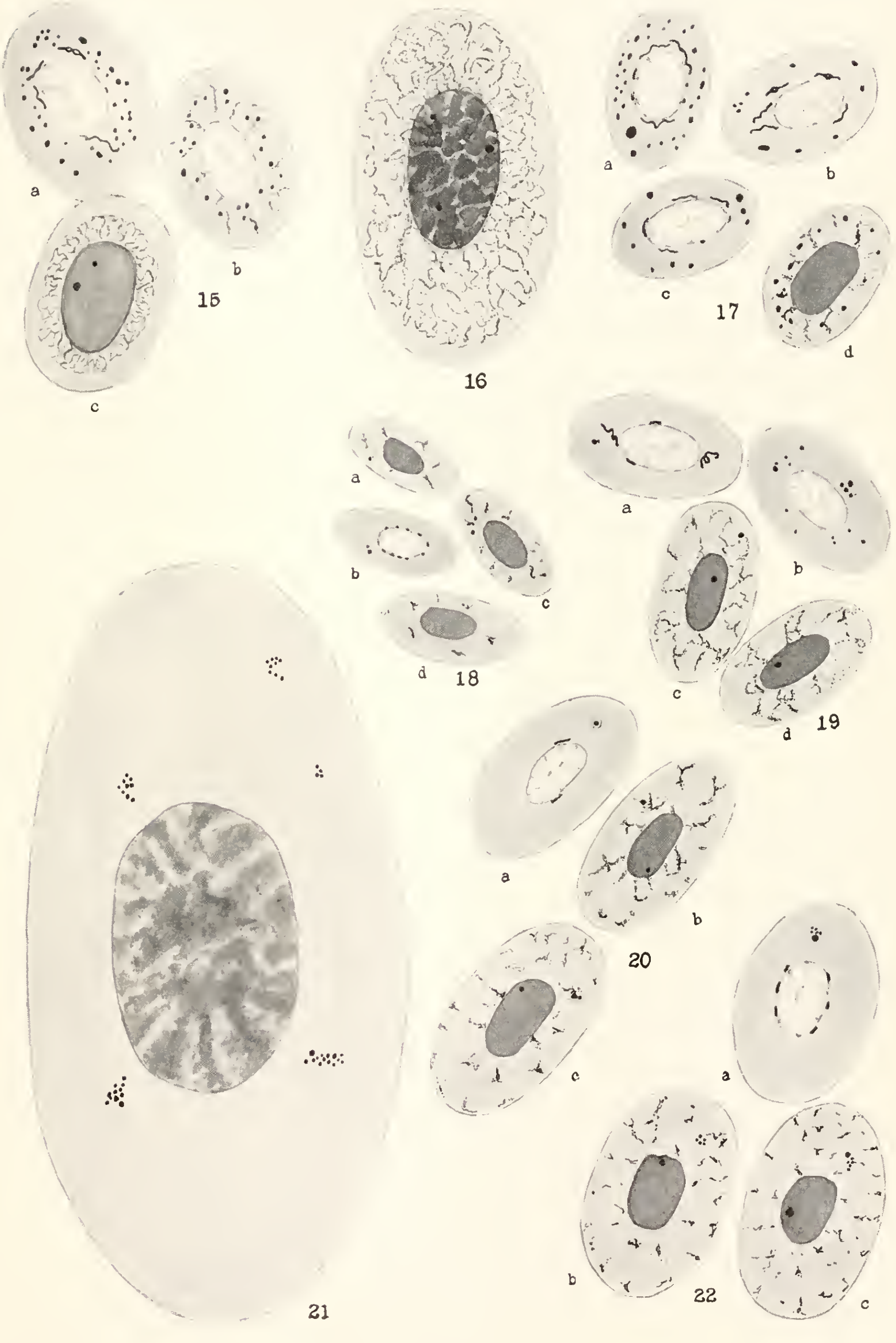
18. Erythrocytes of the domestic fowl showing, (*a, c,* and *d*) reticulation of mature cells; (*b*) primary granules and mitochondria.

19. Erythrocytes of the fence lizard showing, (*a*) primary granules and mitochondria; (*b*) induction of granules after twenty minutes; (*c* and *d*) reticulation and nucleoli in mature cells.

20. Erythrocytes of the horned toad showing, (*a*) primary granule and mitochondria; (*b* and *c*) reticulation and nucleoli in mature cells.

21. An erythrocyte of *Amphiuma means* from a smear stained by Wright's method, showing the primary granules as clusters of basophilic bodies.

22. Erythrocytes of the painted turtle showing, (*a*) the primary granules and mitochondria; (*b* and *c*) reticulation and nucleoli in mature cells.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CYCLOTRICHIUM MEUNIERI SP. NOV. (PROTOZOA, CILIATA); CAUSE OF RED WATER IN THE GULF OF MAINE

PHILIP B. A. POWERS

Zoölogical Laboratory, University of Pennsylvania

I. Introduction

During the warmest days of August, 1931, there were noticed in Frenchman Bay[1] on several occasions, great patches and lanes of red water. This has been known to occur in previous years but rarely so early as the fifth of August. These patches of red water were caused by swarms of small red animals, which appeared in layers from one to three meters beneath the water level. In spots these animals would be more crowded, thus varying the density of the patch as a whole and making the color vary from a brick to a blood red. Between the action of the wind and tide these patches would be streaked out in great lanes and irregular areas. On some days these patches would be more numerous than on others. As to the origin and ultimate fate of these areas of red water, the writer has no information.

A microscopic examination of a drop of sea water containing these organisms revealed hundreds of small red animals which moved rapidly in a zig-zag fashion reminding one of the characteristic movements of *Halteria*. On preliminary examination their appearance suggested that of trochophore larvæ; however, their incredible numbers and small size suggested that they were protozoa. Since the living animals died and disintegrated within one or two minutes upon exposure in a drop of water on a glass slip, no definite conclusion could be reached as to their morphology from a study of the living material.

Further study indicated that these animals were swarms of a ciliate belonging to the genus *Cyclotrichium* Meunier. I have named it *Cyclotrichium meunieri* sp. nov.

The writer wishes to express his appreciation to Mr. William Procter of Bar Harbor, Me., in whose laboratory this study was initiated; and to Dr. D. H. Wenrich for his many helpful suggestions and criticism of the manuscript.

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[1] Frenchman Bay is that part of the Gulf of Maine which separates the northwest side of Mt. Desert Island from the mainland.

II. TECHNIQUE

Quart jars of this red water were collected and allowed to stand in a cold place for about 20 minutes, in which time the organisms settled to the bottom. With a long pipette this sediment was collected and spurted into flasks half filled with warm (40° C.) fixative. Both Schaudinn's and Bouin's fixatives were used. After 30 minutes' fixation the animals were removed from the fixative with the aid of a centrifuge, washed and stored in 70 per cent alcohol. At the close of the summer, vials containing this fixed material were brought back to the University, where the slides were made and the study completed.

By mixing, on a cover-glass, a drop of this alcoholic sediment of fixed organisms with an equal amount of Mayer's egg albumen, spreading it carefully so that it would dry slightly, and finally flooding the cover-glass with absolute alcohol, these minute organisms were fastened to the cover-glass to facilitate their easy manipulation during staining. After 15 minutes in absolute alcohol the cover-slips were gradually transferred to water. Part of the material was stained with iron hæmatoxylin, Mayer's hæmalum, or Delafield's hæmatoxylin; and part by Feulgen's nucleal reaction.

It was found necessary to section some of this material, and in order to handle these small animals in paraffin a mixture of the alcoholic sediment and Mayer's egg albumen was placed in a concavity cut in a small block of preserved (70 per cent alcohol) liver, coagulated with absolute alcohol; and this block, with embedded protozoa, was then handled as a piece of tissue. By infiltrating in 67° paraffin and treating the block with ice water before cutting, sections of 4 μ could be cut with ease. The same stains were used for the sectioned material as for the whole mounts, with the exception that counterstains of orange G or eosine were used to demonstrate the cilia.

III. OBSERVATIONS

Cyclotrichium meunieri (Fig. 1), is almost oval, with the anterior end blunted and the posterior region slightly conoid.

Size.—This ciliate is relatively small. Twenty-five specimens selected at random from fixed material averaged 33 μ (25–42 μ) in length and 22 μ (18–34 μ) in width through the anterior region, whose diameter is somewhat greater than that of the posterior portion.

Cytostome.—The cytostome could not be definitely located; however, most of the specimens show, at the larger end, a depression (Figs. 1 and 2) slightly funnel-shaped and leading into the interior without any well-marked structures. This location of the cytostome agrees in general with that described by Meunier (1910) for *C. cyclokaryon;* and with descriptions given of the cytostomal regions of a number of species of *Cyclotrichium* described by Fauré-Fremiet (1924).

Ciliary Band.—A broad band of cilia is found about the middle. The fine, closely-set cilia are from 5 to 6 μ long and are found only in the depressed mid-region or ciliary band. Besides being depressed and ciliated, this band is further indicated by 52–60 striations which run parallel to the longitudinal axis. Each striation is made up of 5 to 9 granules; anteriorly the granules form a definite ring (Figs. 1 and 3, *A. G. R.*), while posteriorly they become slightly larger (Fig. 1). With iron hæmatoxylin these granules stain black, as do the metaplasmic granules of the endoplasm. With Delafield's hæmatoxylin or Mayer's hæmalum the metaplasmic granules fail to stain while the granules of the *ciliary band* are definitely outlined, thus indicating that the two types of granules are not the same. Because of the close association

EXPLANATION OF PLATE

Cyclotrichium meunieri sp. nov. All drawings have been made at a magnification of 2900 diameters and reduced about two-fifths in printing. The animals have been fixed with Bouin's fluid, stained in Heidenhain's hæmatoxylin, with the exception of those shown in Figs. 7 and 8 which were treated for the Feulgen reaction.

Abbreviations

A.G.R., anterior granular ring
B.G., basal granules
C., cilia
C.B., ciliary band
C.Bu., ciliary bundles
C.C., compound cilia
Ch., chromatin
C.P., chromatophore platelets
Cy., cytostome
E., endoplasm with inclusions
E.V., extra-nuclear vesicle
L.S., longitudinal striations
M., macronucleus
m., micronucleus
Mc., metaplastids
P., pellicle
P.B., pyrenoid body
Pl., plastin
S.S., shrinkage space or artifact

Explanation of Figures

1. Side view of *Cyclotrichium meunieri.* Chromatophore platelets and pyrenoid bodies do not stain in the whole mounts.

2. Sagittal section showing arrangement of chromatophore platelets, pyrenoid bodies, etc.

3. Sagittal section through region of ciliary band showing the arrangement of the basal granules.

4. A diagram of the possible arrangement of cilia in compound units to explain structures as shown in Fig. 6.

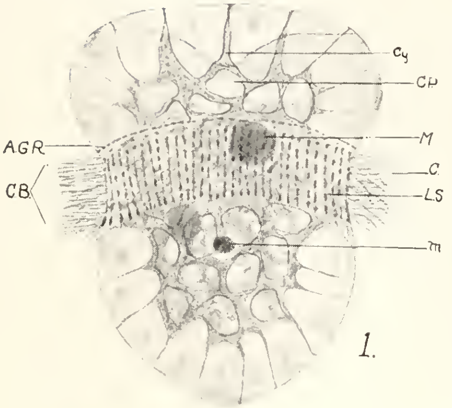
5. Cross section through anterior region.

6. Cross section through region of ciliary band. Cilia are finer and more numerous than could be shown in drawing.

7. Early telophase in dividing individual; six chromosomes are seen in each daughter.

8. Nuclear complexes showing variations. (*a*) micronucleus and two extra-nuclear vesicles; (*b*) micro- and macronucleus and two extra-nuclear vesicles; (*c*) a stage somewhat later than that shown in Fig. 7. Macronucleus is seen in only one of the daughter cells.

PLATE I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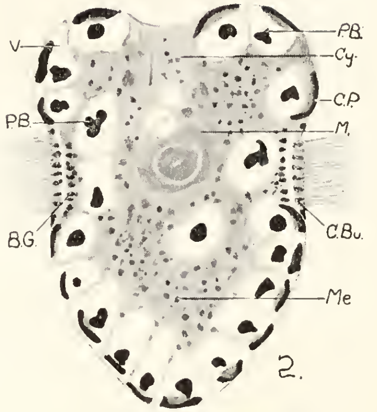
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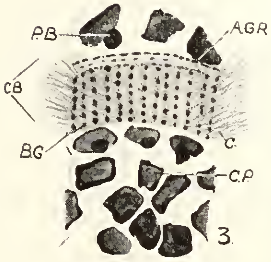
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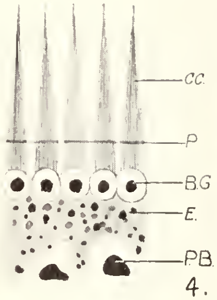

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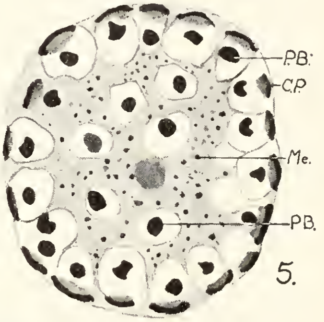

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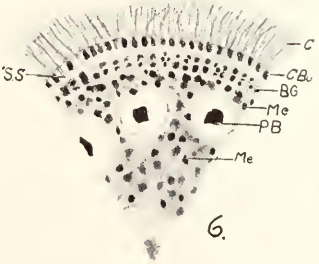
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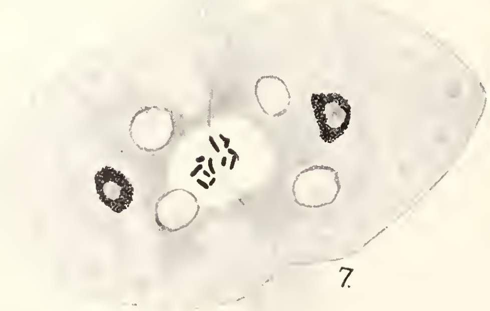

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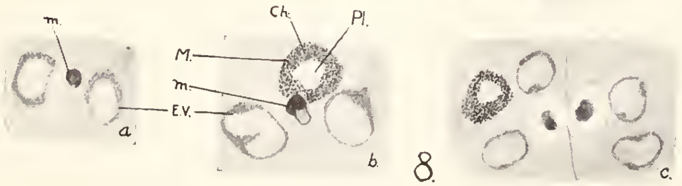
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Ch
Pl.
M.
m.
E.V.
a
b
c

8.

of these granules of the striations with the cilia, I have designated them as basal granules.

The uneven movement of the living animal suggests the presence of cirri or membranelles; however, since the sectioned material showed only these exceedingly fine and numerous cilia and their large associated (compound?) basal granules (Fig. 3, *B. G.*), it would seem that we have in the living condition a system of compound cilia which at death separate into their component units (Fig. 6, *C.*). A diagrammatic interpretation of these compound cilia is shown in Fig. 4. Further evidence for this arrangement is presented by the staining reaction. With iron hæmatoxylin the points of insertion of the cilia stain after the manner of a clump of fibrils (Fig. 6, *C.*, *Bu.*), suggesting definite bunches or bundles of cilia.

Ectoplasm.—Beneath a thin, smooth pellicle, completely shielding the endoplasm, is a series of irregularly concave chromatophore platelets (Fig. 2, *C. P.*), each with an associated pyrenoid body (Fig. 2, *P. B.*); the whole being inclosed by a large vacuole. The red color of *C. meunieri* is doubtless due to the presence of a hæmatochromatous substance localized in the chromatophore platelets, which are possibly of an amylaceous nature. These platelets stain deeply with iron hæmatoxylin but not at all with Mayer's hæmalum. That these platelets have a definite body is demonstrated by the fact that they are often torn from their place due to the impact of the knife during sectioning. In the region of the ciliary band the platelets are missing while the pyrenoid bodies with their vacuoles remain (Fig. 2).

Endoplasm.—After staining sections with iron hæmatoxylin the endoplasm is crowded with many darkly staining bodies. These are designated metaplasmic granules, for they are doubtless associated with the metabolic processes. Because of the abundance of these metaplasmic granules, the nuclear apparatus could be demonstrated successfully only by the aid of the Feulgen technique.

A typical ciliate nuclear complex is present. The macronucleus is slightly irregular and demonstrates a definite core of plastin (Fig. 8*b*, *M.*) surrounded by a layer of granular chromatin. The micronucleus is small, sometimes vesiculated (Fig. 8*b*, *m*). Besides the macro- and micronucleus there are found one or more bodies, irregular in shape and staining but slightly with Feulgen's reagent, which are designated as extra-nuclear vesicles (Fig. 8, *E. V.*). These extra-nuclear vesicles are always present and seem to be either the formative or degenerative stage in the development of the macronucleus.

A number of dividing individuals were studied, and in one (Fig. 7) showing an early telophase stage in the division of the micronucleus six chromosomes could be counted in each daughter nucleus. A somewhat later stage than this is shown in Fig. 8*c*; in this case only one daughter individual received a definitive macronucleus, the other had but the extranuclear vesicles.

Occurrence.—Since single individuals of *C. meunieri* have been found in sea water taken either from the storage tanks of the laboratory, or among the material from plankton hauls; it seems reasonable to look upon this species as a member of the protozoan fauna of this region. Its sudden appearance in swarms among the surface plankton of the bay must be correlated with the periodic enrichment of the water by the nitrogen-bearing algæ whose numbers increase during periods of warmth and excessive sunshine; all of which factors tend to make areas of the bay excellent culture media for these red water ciliates. In the present instance the summer had been warmer than usual, particularly during the last of July and the first week of August.

IV. Discussion

Many organisms are known, under favorable conditions, to multiply in such numbers as to discolor great bodies of water. Martin and Nelson (1929) review this subject and give instances of red water occurring in Delaware Bay due to the swarming of *Amphidinium fusiforme.* Kofoid and Swezy (1921) record the occurrence of swarms of *Gonyaulax polyhedra* as being the most frequent cause of red water along the Pacific coast.

I have placed the ciliate causing red water in the Gulf of Maine in the genus *Cyclotrichium* Meunier because it seems to resemble very closely a ciliate of rare occurrence in the plankton hauls from Barents Sea. Meunier (1910) established this genus for *C. cyclokaryon* but figured a number of ciliates as *Cyclotrichium* Sp? because their poor preservation would not permit further classification. Those ciliates were included in the genus *Cyclotrichium* which had a ciliary band or belt in an equatorial depression which divided the body into two subspherical halves. Making allowance for the poor fixation of his material, the organisms which he described under the name of *Cyclotrichium* Sp? may be likened to those found off the coast of Maine which I have named *C. meunieri.*

The Maine fishermen recognize this red water as a source of food for the herring sardine and it is said that when the red water is present in the herring's intestines they become unfit for sale. Mackerel are also reported by the fishermen as sometimes containing " red feed " in their digestive tracts. It would seem that " red water " and " red feed " are due to two different organisms. Inquiring into this condition further, the writer corresponded with Dr. A. G. Huntsman, Director of the Atlantic Biological Station at St. Andrews, mentioning these occurrences. Dr. Huntsman replied as follows:

" In the St. Andrews region we have never seen the water coloured crimson by the form you mention. In warm summers the warmest strip near the center of the upper end of Passamaquoddy Bay sometimes becomes decidedly reddish and this has been found due to large numbers of different kinds of Tintinnoids which have been studied by Professor J. N. Gowanlock, although his report has not yet been published. In the St. Andrews region the term ' red feed ' is given to copepods as they occur in the stomach of herring, particularly the young herring or sardine. The most abundant form is *Calanus* and at times the swarms of this species give a reddish cast to small areas of the water."

To my knowledge *Cyclotrichium meunieri* is the first holotrichous ciliate to be associated with the appearance of red water in the ocean.

V. SUMMARY

1. Red water in Frenchman Bay is caused by the swarming of a small red ciliate, *Cyclotrichium meunieri* sp. nov.

2. This organism is ovoid, about 33 μ long, and has in a wide depression about its middle a band of fine cilia. It has been suggested that compound cilia may be present in the living animals. The endoplasm is well shielded by a peripheral series of chromatophore platelets, each with an associated pyrenoid body.

3. Six chromosomes were observed in each of the daughter micronuclei in an early telophase of division.

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THE NUMBER OF PRE-ADULT INSTARS, GROWTH, RELATIVE GROWTH, AND VARIATION IN DAPHNIA MAGNA

BERTIL GOTTFRID ANDERSON[1]

Biological Laboratory, Western Reserve University

Until recently growth and variation in Crustacea have been studied by means of preserved materials. These materials may consist of a single collection taken in a certain locality at a specific time or several collections made in different localities at different times. Often conclusions are drawn from an isolated collection. Such studies necessarily neglect the past history of the individual. Any conclusions reached by such methods regarding growth and variation can only be regarded as tentative and subject to verification by experiments with individually reared animals.

As a result of such studies on *Coronis*, Brooks (1886) was led to the statement: " . . . the length of the larva increases uniformly at each moult by one fourth of its length before the moult." Later Fowler (1909) designated this numerical relation " Brooks' Law." He also called the fixed fractional increase the " growth factor."

That " Brooks' Law " does not hold for Cladocera has been shown by Rammner (1930) after a series of studies on individually reared animals from several genera. Gurney (1929) doubts that the above relations exist for copepods. Other arthropods as well do not follow " Brooks' Law." Calvert (1929) found that larval Odonata grow quite irregularly.

Such studies with Cladocera have perhaps led to the conclusion that individuals of a species pass through a definite number of pre-adult instars. When a graph of a population is made wherein the number of individuals of a size class is plotted against size, *i.e.*, total length, a series of size modes is secured (Fig. 1). These size modes are taken as representative of the growth stages of the organism. Usually females of a given species which are of a specific size or larger bear eggs in their brood chambers. The number of size modes between the embryonic stages and the mode of the smallest egg-bearing females is taken as the number of pre-adult instars for the species.

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[1] A part of the experimental work related in this paper was done at the Zoölogical Laboratory of the State University of Iowa.

Inspection of graphs of size distributions such as in Fig. 1 shows that the size groups represented by the modes are not entirely distinct. The size groups in the lower size ranges are usually quite distinct but not decidedly disjoined from each other. Those in the higher size ranges tend to run together and are not so easily identified. Since the size groups are not entirely distinct over the pre-adult range and the past history of each individual is not known, the conclusion that all individuals of a species have a definite number of pre-adult instars is not necessarily valid.

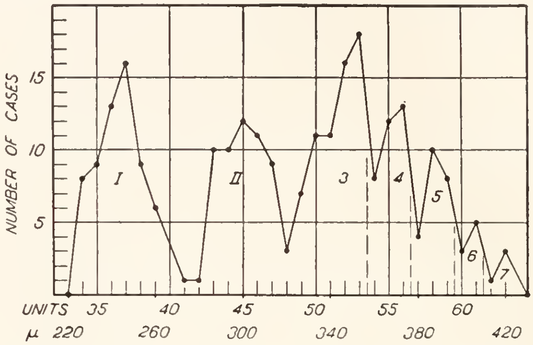

Fig. 1. Distribution of a population of *Chydorus sphæricus* according to length (after Werner).

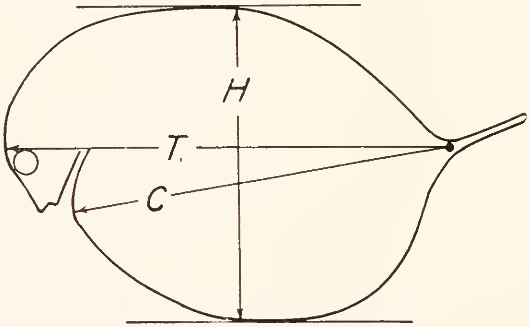

Fig. 2. Diagram showing methods of making measurements. *T*—total length, longest dimension of animal exclusive of spine. *C*—carapace length, longest dimension of carapace exclusive of spine. *H*—height, the shortest distance between two lines tangent to the carapace, as illustrated, and parallel to the line of *T*. This measure of height is affected but little by the number of young in the brood chamber.

The writer finds that the number of pre-adult instars for *Daphnia magna* is rather variable. Calvert (1929) reported that the number of larval instars for certain species of Odonata is not constant. Dover (1931) reported that the number of moults in *Orgyia turbata* decreases when fed on *Crotolaria.* Singh-Pruthi (1925, etc.) found that the number of moults for mealworms can be varied. Schubert (1929) examined *Ceriodaphnia reticulata* from two ponds. The material from the one pond was collected early in July, 1926. He believed that all *C. reticulata* in this collection were primiparous in the fourth instar. The material from the second pond was taken in mid-September of the same year. His findings regarding the latter were that 60 per cent of the individuals were primiparous in the third instar and that 16 per cent were still immature in the fourth. He concluded that at least two varieties of *C. reticulata* exist in these ponds. Apparently the number of pre-adult instars is variable in this species. Agar (1930) reports that *Simocephalus gibbosus* and *Daphnia carinata* have three and four pre-adult instars respectively but adds that a small percentage of the individuals become mature in one less than the average.

The aim of this paper is to present data on growth of individually reared *Daphnia magna* and further to consider the application of the equation

$$y = bx^k$$

to relative growth and variation in Cladocera.

MATERIALS AND METHODS

Female *Daphnia magna* Straus. of several clones were employed. One clone was used for the major portion of the work. To determine whether or not the observed results were characteristic only of the one clone, some six others were tested. One of the latter was secured from Dr. A. M. Banta. All of the other clones, including the one first mentioned, were derived from ephippial eggs which may be traced to Banta's stocks.

Individual females were isolated within six hours of their release from the brood chambers of the mothers. These were measured and placed in separate vials containing thirty to thirty-five cubic centimeters of a culture medium. Banta's manure-soil medium (Banta, 1921) and oatmeal and wheat modifications were employed. More uniform results were obtained from the regular manure-soil medium than from modifications, as the results of one series of experiments indicate.

Where a modified culture medium was used, the animals were placed in old manure-soil medium and a drop of an oatmeal mixture or wheat infusion was added daily. Water was added to replace the fluid lost by evaporation. The oatmeal mixture was prepared by cooking rolled oats for a half hour in about twice the amount of water ordinarily used in making a porridge. The mixture was then strained through gauze and kept in a refrigerator until used. The wheat infusion was prepared by

TABLE I

Mean total lengths of female *Daphnia magna* of one clone during each of the pre-adult instars and the first adult for different classes of individuals. The classes are based on the number of pre-adult instars and the nature of the culture medium.

MEDIUM	MANURE-SOIL	OATMEAL MODIFICATION				
Number of Pre-adult Instars	5	5	6	7	8	
Number of Cases	19	8	29	39	5	
First Instar	0.85 ± 0.01	0.80 ± 0.01	0.84 ± 0.01	0.83 ± 0.01	0.77 ± 0.02	MEAN TOTAL LENGTH IN MM.
Second Instar	1.09 ± 0.02	1.02 ± 0.01	1.00 ± 0.01	0.94 ± 0.01	0.86 ± 0.02	
Third Instar	1.39 ± 0.02	1.33 ± 0.03	1.17 ± 0.01	1.05 ± 0.01	0.99 ± 0.03	
Fourth Instar	1.77 ± 0.02	1.70 ± 0.03	1.36 ± 0.02	1.19 ± 0.01	1.12 ± 0.04	
Fifth Instar	2.13 ± 0.02	2.06 ± 0.04	1.64 ± 0.02	1.38 ± 0.01	1.22 ± 0.05	
Sixth Instar	Adult 2.60 ± 0.02	Adult 2.56 ± 0.03	1.99 ± 0.02	1.69 ± 0.01	1.55 ± 0.10	
Seventh Instar			Adult 2.49 ± 0.02	2.08 ± 0.03	1.87 ± 0.09	
Eighth Instar				Adult 2.56 ± 0.03	2.20 ± 0.04	
Ninth Instar					Adult 2.52 ± 0.03	
Number of Young in First Brood	6.4 ± 0.3	6.4 ± 0.6	7.3 ± 0.2	8.6 ± 0.3	5.8 ± 0.8	
Number of Young in Second Brood	9.5 ± 0.5	9.4 ± 0.6	7.4 ± 0.3	7.1 ± 0.2	5.2 ± 1.0	

boiling wheat in water, decanting the fluid, and allowing this to stand open for several days before using. When the manure-soil medium was used exclusively, one-third of the fluid was removed semiweekly from each vial and replaced by fresh medium.

At the time of isolation of the individuals and after every moult each individual was placed in a watch glass together with a few drops of the culture medium. Just enough of a saturated solution of chloretone was added to bring about cessation of movement. The chloretone did not appear to have any detrimental effects. By means of an ocular micrometer the measurements as illustrated in Figure 2 were made. The three measurements consisted of total length exclusive of the spine, carapace length, and height. Camera lucida outline drawings were made during each instar for each of three animals. In addition to these measurements, note was taken as to the presence of eggs in the brood chamber. Within twenty-four hours after their release the young were removed from the vials and counted.

Size and shape change only at the time of moulting in this species. Repeated measurements of any dimension during a single instar always gave the same results. Agar (1930) pointed out this fact for *Daphnia carinata* and *Simocephalus gibbosus.* Such being the case, a single set of measurements suffice for each animal during any one instar.

All the experiments were carried out at room temperature (18°–23° C.).

Number of Pre-Adult Instars and Growth

The results of observations on the number of pre-adult instars, *i.e.,* the number of instars elapsing between the time of release of the individual female from the brood chamber of her mother and the appearance of eggs in its own brood chamber, and the mean total length of the animals during each are summarized in Table I. The data were segregated on the basis of the number of pre-adult instars and the culture medium used. The number of pre-adult instars varied for the animals reared in the oatmeal modification. For those reared on the standard manure-soil medium the number was constant. Twenty individuals only were used in the latter series, one of which died before reaching maturity.

The number of pre-adult instars above five may perhaps be explained on the basis of food deficiency. Great numbers of large ciliates were found in the modified medium. These probably reduced the number of smaller microörganisms that ordinarily would have been available as food to the *Daphnia.* The ciliates themselves were too large to be consumed. Conditions similar to this arose at a time when great numbers of the annelid *Aeolosoma* were found in the culture medium. At times

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a culture medium seems to stimulate the adult females to produce great numbers of young, but this same lot of medium does not seem sufficient for the development of the young.

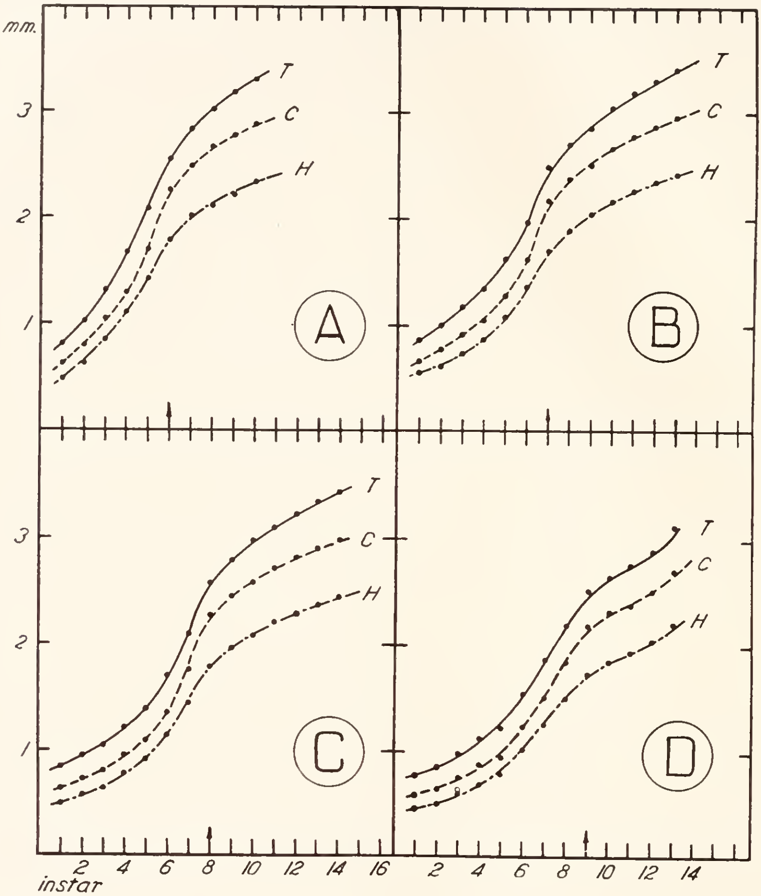

FIG. 3. Growth curves of different classes of individuals reared on the oatmeal modification; *A*—those animals with five pre-adult instars (7 individuals), *B*—six (18 individuals), *C*—seven (30 individuals), *D*—eight (5 individuals). *T*—total length; *C*—carapace length; *H*—height. The arrow indicates the first adult instar.

The animals used in the experiment with the modified medium were all released on the same day. The time taken to reach sexual maturity varied from six to ten days. The number of days corresponded approximately to the number of pre-adult instars in each case. The animals were all kept under identical conditions and the culture medium was from the same lot. The experiment using the standard manure-soil medium was begun several weeks later.

Table I brings out an interesting relation. The mean size for each group during the instar when eggs first appear in the brood chamber varies from 2.49 mm. to 2.60 millimeters. Apparently *Daphnia magna* must attain a certain size before becoming sexually mature. Singh-Pruthi (1925, etc.) found that metamorphosis of mealworms took place only after the larvæ were full-grown.

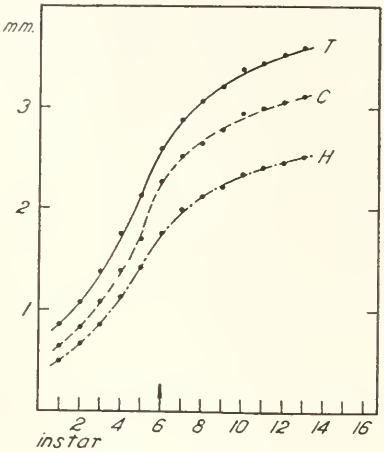

Fig. 4. Growth curves of a group of animals reared on unmodified manure-soil medium. These had five pre-adult instars (7 individuals). *T*—total length; *C*—carapace length; *H*—height. The arrow indicates the first adult instar.

Rammner (1930*b*) has found that eggs were produced after the fourth instar in *Daphnia magna*. After observation on well over a thousand individually reared females, the writer has never observed less than five. L. A. Brown has observed five pre-adult instars for this

Table II

Number of Individuals Primiparous During the Sixth to Ninth Instar

Clone	Sixth Instar	Seventh Instar	Eighth Instar	Ninth Instar	∞
B	4	10	4	2	0
C	8	3	1	0	1
D	7	4	0	0	0
E	9	1	1	0	0
F	6	11	2	0	0
G *	10	4	4	0	1

∞ Those living beyond the 10th instar without bearing eggs.
* Clone secured from Dr. A. M. Banta.

species (unpublished data). Rammner's smaller number may be due to some factor in the culture medium or perhaps to a genetic difference in his animals.

Figures 3 and 4 are growth curves constructed from data secured from animals included in Table I. The data used were taken only

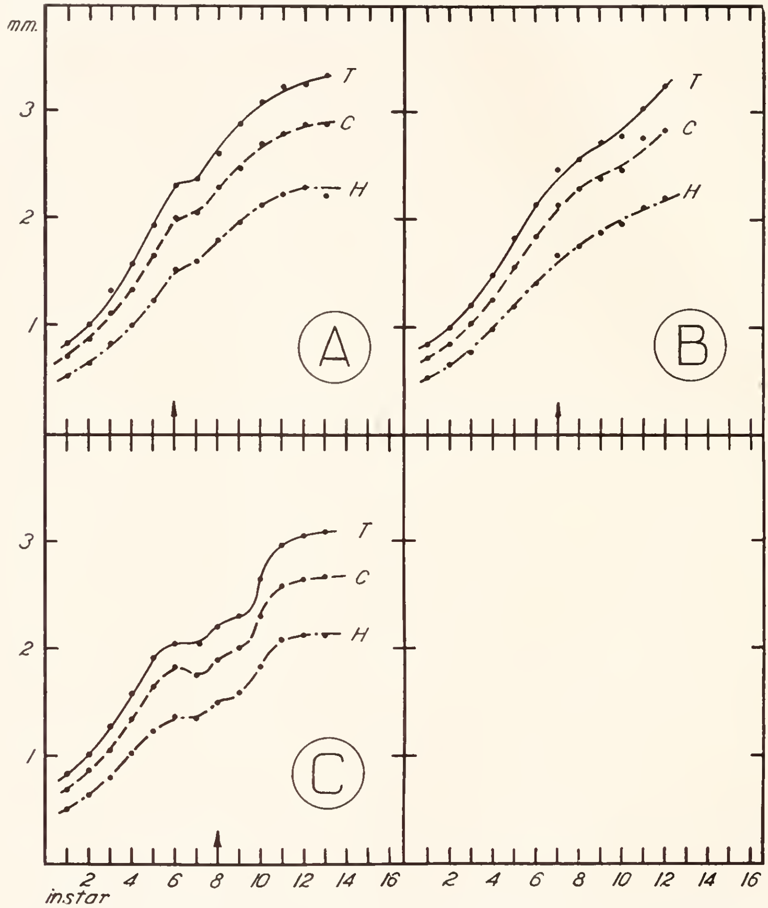

Fig. 5. Growth curves of three broodmates of clone *B*. Animal *A* had five pre-adult instars; *B*, six; and *C*, seven. *T*—total length; *C*—carapace length; *H*—height. Arrow indicates first adult instar.

from those animals which were still living during the last instar recorded on the graphs. All such are included. The inflection in the curves always occurs at a point which corresponds to the time of sexual maturity.

All the individuals used in the above experiments were of the same clone. To determine whether or not the variation in the number of pre-adult instars was characteristic only of this clone, another series of experiments was performed using six other clones. Five of these were raised from ephippial eggs. A sixth clone was secured from Dr. A. M. Banta. Manure-soil medium and the wheat modification were employed in these tests. Table II gives the results. Examination of this table shows that the variability in the number of pre-adult instars is characteristic of all clones used. Variation occurred to about the same extent in both media.

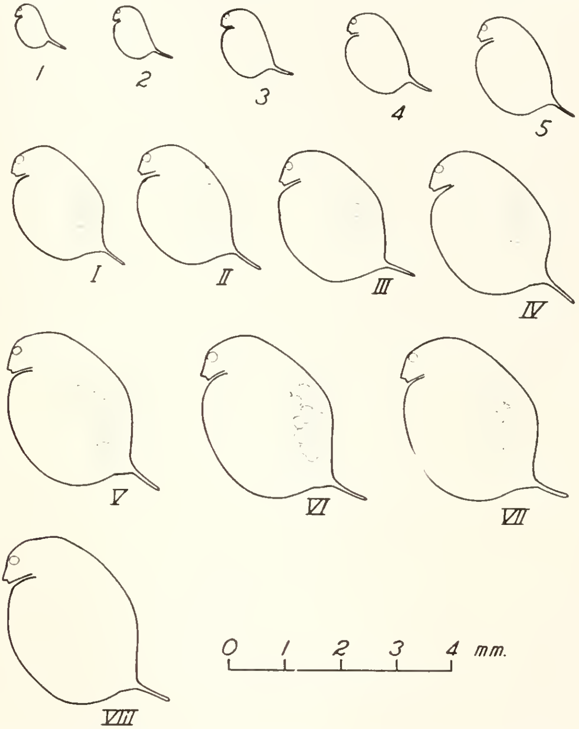

FIG. 6. Outline drawings of a single animal during each of the first thirteen instars. The animal is the one for which growth curves are shown in Figure 5*A*. The arabic numerals designate pre-adult instars; the Roman numerals—adult instars.

Two of the animals included in the above experiments lived for at least thirteen instars without bearing young. Ordinarily the ovary in the adult assumes a green color, especially toward the end of the instar. Microscopic examination did at no time reveal the development of the green color in the ovaries of either animal. In one of them growth did not seem retarded. Her total length during the thirteenth instar equaled the average total length, during the same instar, of those primiparous in the sixth.

Individual growth curves for three brood mates of clone *B* are shown in Figure 5. Each of these as designated *A*, *B*, and *C*, bore its first clutch of eggs in the sixth, seventh, and eighth instars respectively. These were raised under the same conditions and at the same time, using the same lot of unmodified manure-soil medium. The measurements for these were made by means of camera-lucida drawings. Figure 6 is a reproduction of the series of camera-lucida drawings for animal *A*.

TABLE III

Growth ratios for various classes of animals according to the nature of the culture medium, number of pre-adult instars, and clone

Instars	CLASS *							
	M6	M7	M8	M9	R6	B6	B7	B8
1– 2	1.27	1.16	1.13	1.11	1.26	1.24	1.19	1.25
2– 3	1.29	1.17	1.10	1.15	1.29	1.28	1.21	1.23
3– 4	1.27	1.14	1.17	1.14	1.27	1.19	1.23	1.24
4– 5	1.25	1.21	1.14	1.09	1.22	1.23	1.23	1.21
5– 6	1.24†	1.21	1.22	1.27	1.22†	1.19†	1.18	1.07
6– 7	1.10	1.26†	1.24	1.21	1.11	1.02	1.15†	1.00
7– 8	1.07	1.09	1.23†	1.18	1.07	1.10	1.04	1.08†
8– 9	1.05	1.06	1.08	1.15†	1.05	1.11	1.07	1.05
9–10	1.04	1.07	1.06	1.05	1.05	1.07	1.02	1.15
10–11		1.05	1.04	1.05	1.02	1.05	1.09	1.12
11–12		1.03	1.04	1.05	1.02	1.01	1.07	1.03
12–13		1.03	1.05	1.08	1.02	1.02		1.01
13–14			1.03					
Number of Animals	7	18	30	5	7	1	1	1

* Classes—M6, M7, M8, and M9 from clone *A* reared on modified medium and primiparous in the sixth, seventh, eighth, and ninth instars respectively. R6 from clone *A* and reared on the regular manure-soil medium, primiparous in the sixth instar. B6, B7, and B8 from clone *B*, primiparous in the sixth, seventh, and eighth instars respectively. This table is based on the same data as are Figs. 3, 4, and 5. The ratios are based on total length.

† Denotes instar during which animals were primiparous.

The irregularities in the number of pre-adult instars and in the growth of *Daphnia magna* as brought out above indicate that no law of growth such as " Brooks' Law " is valid under all circumstances. Table III brings the case out more clearly. This table gives the values of the ratio of the total length during each instar for each group to that of the previous instar. These values correspond to the "Wachstumsquotienten" of Rammner (1930) and others. For those cases in which the individuals were primiparous in the sixth instar the value is fairly constant during the pre-adult stages. In all other instances the value is quite variable. After sexual maturity the value decreases considerably and approaches unity in old age. The validity of "Brooks' Law" with regard to *Daphnia magna* may therefore be considered to depend on the conditions of the individual and of the environment.

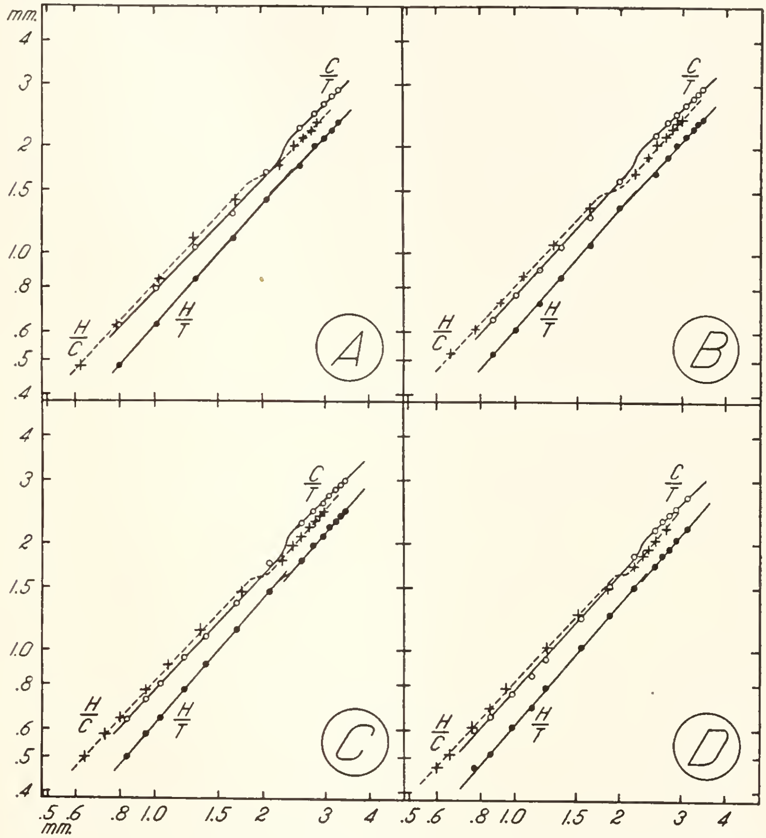

FIG. 7. *Log log* plots of the relations between carapace length and total length C/T, height and total length H/T, and height and carapace length H/C during each instar for different classes of animals reared on the oatmeal modification—the same for which growth curves are shown in Figure 3. The lines were drawn according to the calculated values of the constants given in Table IV. The breaks in the relations are coincident with sexual maturity. *A*—animals with five pre-adult instars (7 individuals), *B*—six (18 individuals), *C*—seven (30 individuals), and *D*—eight (5 individuals).

Relative Growth and Variation

The foregoing portion of this paper has brought out the wide irregularities in the general growth rate of individual *Daphnia magna.* In spite of these irregularities the relative growth of parts is quite constant. If the logarithm of the carapace length be plotted against the logarithm of the total length for each, two straight lines may be drawn—one through the points for the pre-adult instars and the other for the adult instars. The lines so drawn differ slightly in slope and in position (Figs. 7, 8, and 9). Such *log log* plots of height against total length and against carapace length show similar characteristics. These linear relations can be expressed as

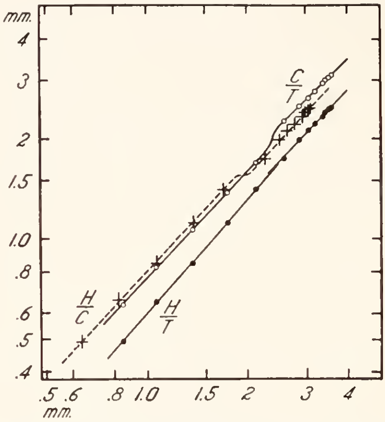

Fig. 8. *Log log* plots of the relations between carapace length and total length C/T, height and total length H/T, and height and carapace length H/C during each instar for a group of animals reared on unmodified manure-soil medium—the same for which growth curves are shown in Figure 4. The lines were drawn according to the calculated values of the constants given in Table IV. The breaks in the relations are coincident with sexual maturity.

$$y = bx^k,$$

where b is a constant and k is the "differential growth ratio" of y compared with x. Huxley (1924, etc.)[2] has found that a large number of organs obey this law. Hersh (1928, 1931) has shown that the facet numbers in the dorsal and ventral lobes of bar-eyed *Drosophila* conform to this equation. Robb (1929) has applied this relation to relative growth of organs in mammals and found it satisfactory.

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[2] For an elucidating treatise on relative growth see Huxley "Problems of Relative Growth" (Methuen Co., London, 1932). This work became available to the author after having submitted the present paper to the editors.

Table IV

Values of the constants *b* and *k* during pre-adult and adult instars for various classes of *Daphnia magna* based on the nature of the culture medium, number of pre-adult instars, and clone. These relations are shown graphically in Figs. 7, 8, and 9.

	TOTAL LENGTH (x) CARAPACE LENGTH (y)				TOTAL LENGTH (x) HEIGHT (y)				CARAPACE LENGTH (x) HEIGHT (y)				NUMBER OF ANIMALS
	Pre-adult		Adult		Pre-adult		Adult		Pre-adult		Adult		
Class*	k	b	k	b	k	b	k	b	k	b	k	b	
M6	1.03	0.78	0.99	0.88	1.12	0.62	1.03	0.67	1.09	0.81	1.04	0.77	7
M7	1.07	0.77	0.96	0.91	1.15	0.62	1.07	0.65	1.08	0.82	1.12	0.72	18
M8	1.08	0.77	1.02	0.86	1.16	0.62	1.15	0.60	1.08	0.82	1.13	0.71	30
M9	1.08	0.77	0.95	0.92	1.14	0.62	1.14	0.61	1.06	0.81	1.20	0.68	5
R6	1.06	0.76	0.97	0.90	1.13	0.60	1.05	0.65	1.07	0.80	1.08	0.73	7
B6	1.01	0.84	1.02	0.85	1.01	0.63	1.06	0.64	1.00	0.75	1.04	0.76	1
B7	1.02	0.85	1.07	0.82	1.05	0.64	1.10	0.62	1.03	0.76	1.03	0.76	1
B8	1.07	0.83	1.01	0.86	1.10	0.61	1.05	0.66	1.03	0.75	1.05	0.77	1

* Classes—M6, animals of clone *A* raised on modified medium and primiparous during the sixth instar; M7, M8, M9, as M6 but primiparous during the seventh, eighth, and ninth instars respectively. R6, animals reared on the regular manure-soil medium and primiparous during the sixth instar. B6, B7, B8, individuals of clone *B* primiparous during the sixth, seventh, and eighth instars respectively. The classes are the same as those in Table III and in Figs. 3, 4, 5, 7, 8, and 9 and the values of the constants are based on the same data.

An interesting feature coming out of this study is that relative growth changes at sexual maturity. This may be seen on examination of any one of the *log log* graphs. The change is much more distinct in clone *A* (Figs. 7 and 8). The change in clone *B* is less discernible (Fig. 9). Table IV gives the values of the constants *b* and *k* for both clones. These were computed by the method of averages. Since all points on any one graph represent the same number of cases, the cal-

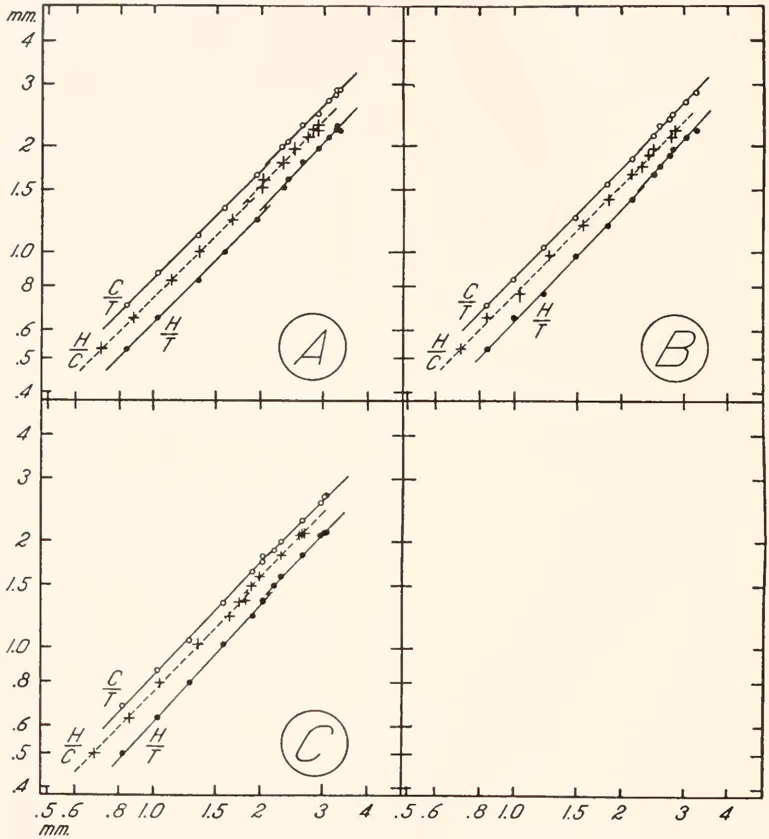

FIG. 9. *Log log* plots of the relations between carapace length and total length *C/T*, height and total length *H/T*, and height and carapace length *H/C* during each instar for three individual broodmates of clone *B*—the same for which growth curves are shown in Figure 5. The lines were drawn according to the calculated values of the constants given in Table IV. The breaks in the relations are coincident with sexual maturity. Animal *A* had five pre-adult instars, *B* six, and *C* seven.

culated values of the constants are practically as accurate as those secured by a more elaborate method.

Huxley (1927) has suggested that heterogony in *Maia* begins at sexual maturity. He also noted that this is probably true for male *Gammarus*. Examination of Figure 1 in the same report shows a change in relative growth of the large chela as against the rest of the body in *Uca pugnax*. Huxley makes no mention as to its significance. Robb (1929) has pointed out that a change takes place in the relative growth of various organs and suggests that another factor becomes involved. These changes are apparently coincident with sexual maturity.

Since the value of k approximates unity for all above relations, both for pre-adult and adult instars of *Daphnia magna,* and the value of b for each relation varies only over a small range, the animal does not change in proportions, to any great extent, during growth. Wesenberg-Lund (1926) writes that variations in *D. magna* of Denmark are insignificant. This condition is therefore as expected.

In a great many Cladocera pronounced variations occur. Rammner (1927) has discussed several methods for their study. As far as the writer is aware, no one has considered applying the equation

$$y = bx^k$$

in such studies.

Masses of data on variation have been collected by various workers on several species (Werner, 1924; Rammner, 1926; Heberer, 1928). The data as presented by these workers do not allow of the proper manipulation for testing the validity of the equation for the purpose. Were the original data available, a satisfactory test might be made. Rammner's (1928) data on individually reared *Chydorus sphæricus* and *Pleuroxus trigonellus* seem quite irregular. Woltereck's (1925) data on individually reared *Daphnia cucullata* seem satisfactory for the pre-adult instars. In *log log* plots of the head length against the body length a break in the relations appears in about the middle of the pre-adult instar range and another at sexual maturity. The first change seems likely to affect only the value of the constant b in the equation while k remains approximately the same. The second change would probably affect the values of both constants. The values of the constants for the adult instars cannot be determined, since the data includes measurements for only one and sometimes two adult instars. Different varieties give different values for the constants. The general trend of the relations is the same.

The advantage of applying the equation

$$y = bx^k$$

to data on variation in Cladocera is that the direction of variation may be expressed numerically in the constants b and k. The data employed could be taken from preserved material such as that used by Werner and others. Huxley and his coworkers have used this method in the study of heterogeny in Crustacea with apparent success. Collections from several localities, as well as those made in the same locality at different seasons, might thus be readily analysed and compared.

The author wishes to express his appreciation to Drs. J. H. Bodine of the State University of Iowa, L. A. Brown of George Washington University, and A. H. Hersh of Western Reserve University for their many helpful suggestions and criticisms, and especially to Dr. Hersh for suggesting the method of treatment of relative growth.

SUMMARY

Observations as to the number of pre-adult instars have been made on over 200 individually reared female *Daphnia magna* of seven clones. In all cases measurements of the total length during each instar were taken. For well over a hundred individuals measurements of carapace length and height were taken in addition to those of total length.

The number of pre-adult instars varied upward from five. This variation was found in all clones tested.

Growth curves have been constructed for various groups based on the number of pre-adult instars. The inflection in the growth curve of any dimension in any group coincides with the time of sexual maturity.

" Brooks' Law " holds only for those groups which were primiparous during the sixth instar and then only approximately.

Relative growth in the dimensions studied, x and y, may be expressed by the equation

$$y = bx^k.$$

Relative growth changes at sexual maturity, *i.e.*, a change occurs in the values of the constants b and k.

The above equation may perhaps be used to advantage in the study of variation in other Cladocera. By the use of this equation the direction of variation can be given numerical values. Comparisons of different races and varieties, both seasonal and geographic, may be readily made on this basis.

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SOME EFFECTS OF HIGH PRESSURE ON DEVELOPING MARINE FORMS

JOHN W. DRAPER AND DAYTON J. EDWARDS

(From the Department of Physiology, Cornell University Medical College, New York City, and the Marine Biological Laboratory, Woods Hole, Mass.)

The action of hydrostatic pressure, below certain limits, in producing an increase in the contractility of cardiac and skeletal muscle, raises a question of prime interest as to the nature of the effect. Does an increase in the absolute pressure of the environment of a tissue, with the resulting increase in tissue density, give rise to a general stimulation of the fundamental processes in the cells? Several lines of approach have been considered for an answer to this question, but the aim in the present experiments will be to deal only with the influence of pressure on certain of the fundamental processes in the early development of marine eggs.

The experimental observations relate mainly to two features: first, the effect of maintained compression on the rate of development of fertilized eggs, particularly in the early stages; and second, the effects produced by pressure on the rate of the heart in embryos at the stage when pulsations are just beginning and then later when the rhythm is fully established.

The observations were made in an apparatus having essentially the same construction as that previously described (Edwards and Cattell, 1928). The fertilized eggs of *Fundulus* were placed in glass vials filled with sea water and covered with thin rubber membrane to prevent their escape. One lot of the eggs was then placed in the compression chamber, which was also filled with sea water, and another lot, the control sample, was placed in a second chamber similar to the first. In this manner the factors of temperature, respiration and amount of agitation were kept constant, and the experimental sample differed only in having pressure applied for known intervals.

In a few experiments the technic was modified to permit observation of the eggs during the period when they were under the action of pressure. The essential things for this purpose consisted of a heavy glass window mounted in one end of the compression chamber, which made it possible to view with low magnification objects placed immediately beneath it, and a depression slide fixed in position close to the inside surface of the window to contain the eggs. A small mirror backing the slide and a " Pointolite " lamp, which directed a beam of light through the window and against the mirror, permitted fair illumination for a microscopic examination of the eggs in the depression of the slide. All of the observations on the change of heart rate under pressure were made by using this adaptation of the pressure apparatus.

An observation of general interest arising from these experiments on compression of the developing egg is the extremely slight change that occurs in the egg structures when subjected to comparatively high pressures. The method has permitted observations on the diameter of the egg, the size and state of aggregation of the fat globules, the size and position of the blastodisc, and the size of the finer blood vessels in parts of the embryo. A close study of these different parts of the developing egg, made during and immediately after the onset of pressure of 110 atmospheres, reveals no significant changes. Observations on advanced embryos within the egg, made while pressures of 1500 pounds per square inch were applied, may reveal nothing more than a few quick movements of the embryo not unlike those shown at indifferent intervals by material of this kind.

Table I

The effect of pressure on the cleavage of Fundulus eggs. The time intervals in the fourth column signify the delay in the development of eggs subjected to pressure as compared with those maintained under control conditions. The observations were made at the cleavage stage shown in the fifth column.

Experiment No.	Pressure	Duration of compression	Delay of pressure eggs	Stage of cleavage
	lb.	*min.*	*min.*	
1	1500	140	15	second
2	1700	60	15	first
			13	second
3	1500	108	20	third
4	1950	120	10	third
5	1950	100	15	first
			15	second

In order to test the effects of pressure on the rate of cell division it was necessary to have some standard for comparing the eggs subjected to pressure with those maintained under control conditions. In our initial experiments the completion of the membrane between two daughter cells was taken arbitrarily as an end-point. With this criterion the data contained in Table I show that the eggs subjected to pressure are delayed about fifteen minutes in reaching a given stage of development. The exact time, however, at which a batch of developing eggs reached a given stage in division was often difficult to determine. Additional observations, therefore, were taken (1) by making counts on the control and the experimental samples to determine the predominant stage at a given time; and (2) by removing samples of 25 or more eggs which were placed in a fixing solution for later examination. The results by these methods of study are set forth in detail in Tables II and III and the evidence they bring forth lends support definitely to the conclusion that compression retards cell division.

The eggs subjected to pressure have been carefully watched for any abnormalities that might occur. A number of monsters have been found of the types showing gross distortions of the body, changes of the cardiovascular system, and tendencies towards anophthalmus. The percentage of abnormal specimens, however, was not large and there was no dominant type of dysmorphism. The abnormalities of the eye were usually those showing one located craniad to the other but not exactly in the midline, while the deformities of the cardiovascular system appeared usually as an asymmetrical development of the vessels. The failure of the blood vascular system to develop symmetrically causes the heart to be drawn to one side of the pericardial cavity.

Table II

The effect of pressure on the cleavage of Fundulus eggs. The data contained in the fourth and fifth columns represent simultaneous observations on samples of eggs subjected to pressure (pressure sample) and samples maintained for the control. The observations were made on living material.

Experiment No.	Pressure	Duration of compression	Pressure sample	Control sample
	lb.	*min.*	*division stage*	*division stage*
1	1300	68	1-cell	2-cell
2	1700	112	2-cell	4-cell
3	1650	60	1 and 2-cell	2 and 4-cell
4	1650	30	1-cell	2-cell
5	1575	180	2-cell	4-cell
6	1560	130	1-cell	2-cell
7	1525	140	1-cell	2-cell
8	1300	70	1-cell	2-cell
9	1500	108	2-cell	4-cell
10	1950	120	4-cell	8-cell

The *Fundulus* embryo is a favorable object for the study of the rate of the heart, since the pulsations of this organ may be observed almost at the time automaticity starts and may be followed until a completely functioning circulatory system is established. As heart automaticity is a fundamental property in development, with a fairly definite time of appearance, the study of the influence of pressure on this phenomenon presented features of unusual interest. The results of 17 experiments are summarized in Table IV. These data show that a pressure of 1200 pounds produces a reduction in the heart rate within two minutes, amounting to an average decrease of 9.9 per cent from the control rate. The decline in rate continues, however, somewhat more slowly than the initial change, so that at the end of a 10-minute period of compression the average reduction was only 16.6 per cent below the control value. When pressure was applied, the pulsations of the heart showed a definite reduction in the rate within a half minute of the onset. Although in some instances a more or less gradual decline prevails under pressure, the more common type of change appears to be a fairly abrupt slowing.

The action of different amounts of pressure was tested, within the range of 400 to 1200 pounds, in an attempt to determine if critical points exist in the pressure effect. The results support the view that the pressure effect becomes progressively greater with the higher grades of compression on the heart. With pressures ranging from 1200 to 1900 pounds we have been able to suppress the automaticity of the heart to the extent of not being able to observe under low magnification any indication of a contractile response. We do not overlook the fact that localized fine pulsations of a few fibers may have persisted in these preparations, so small indeed as to have been beyond our range of identification, but the essential fact is that automaticity was practically stopped. On release of pressure these hearts which have been held quiescent for several minutes immediately show signs of activity and

TABLE III

The effect of pressure on the cleavage of Fundulus eggs. The data contained in the fourth and fifth columns represent counts made on samples of eggs subjected to pressure (pressure sample) and samples maintained for the control. The observations were made on fixed material.

Experiment	Pressure	Duration of compression	Pressure sample			Control sample		
			2-cell stage	4-cell stage	8-cell stage	2-cell stage	4-cell stage	8-cell stage
	lb.	*min.*						
1	1500	135	32	1	0	0	35	0
2	1500	135	30	0	0	0	27	0
3	1500	190	20	0	0	0	20	0
4	1500	175	0	28	4	0	0	32
5	1500	140	13	0	0	0	12	0
Total count			96	29	4	0	94	32

eventually recover, thereby confirming our previous observations (Edwards and Cattell, 1928) that the pressure effect is freely reversible in nature. In establishing a pressure standstill of an embryo heart several changes have been observed to occur, such as arrhythmia, partial and complete heart block, and fibrillary motion of the auricles terminating in a cessation of activity in the region of the sinus. With the reëstablishment of activity after release of pressure, sometimes recovery appeared almost simultaneously in sinus, auricle, and ventricle, while in other instances the return followed the reverse order of the disappearance with some degree of arrhythmia preceding the dominance of the normal type.

Table IV

Effect of Pressure on the Heart Rate in Fundulus Embryo

Experiment	Age	Control heart rate	Pressure	Heart rate during compression		Heart rate after pressure release		Per cent Δ in heart rate during compression	
				2 min.	10 min.	2 min.	5 min.	2 min.	10 min.
	hr.	*beats per min.*	*lb.*	*beats per min.*	*beats per min.*	*beats per min.*	*beats per min.*		
2	120	68	1200	65	65	60	60	7.3	7.3
3	79	62	1200	58	52	53	—	6.4	16.2
4	96	55	1200	47	43	53	50	9.1	21.9
5a	119	53	1200	48	48	55	54	9.4	9.4
5b	120	55	1200	50	48	—	—	9.1	12.8
6	76	55	1200	50	50	—	—	9.1	9.1
7	121	50	1200	44	46	54	—	12.0	8.0
8	192	62	1200	53	53	62	63	14.5	14.5
9	172	57	1200	54	53	56	—	5.2	7.0
10a	216	65	1200	60	59	67	66	7.7	9.2
10b	217	66	1200	60	—	67	67	9.0	—
11	150	73	1200	67	66	72	—	8.2	9.6
17	74	72	1200	69	63	69	72	4.1	12.6
19	26	60	1200	56	50	53	60	6.6	11.7
20	120	60	1200	52	53	64	60	13.3	11.7
21	144	116	1200	96	100	106	103	17.2	13.8
22	144	112	1200	88	84	99	104	21.4	25.1
Average	....	......	....	......	......	......	......	9.9	16.6

The remarkable effect of pressure in reducing and abolishing the rhythmicity of the embryo heart raises a question as to the nature of the action. A factor that immediately occurs to one as a possible cause for the restraining action of pressure is a direct excitation of inhibitory nerve fibers supplying the heart. Experiments designed to throw light on this suggestion were carried out in the following way. Embryos ranging in age from 14 to 20 days were carefully dissected and the heart completely isolated from the surrounding tissue. The preparations were kept immersed in a 40/60 dilution of sea water and tap water to which was added 5 per cent of glucose. These hearts are not easy to handle on account of the very small size, but with care it was possible to mount them on a depression slide and to place them in the compression chamber where their activity could be followed through the window with a low power objective.

The results of these observations confirm in all essentials those obtained with the entire embryos. Moreover, the same type of changes was present, as, for example, the arrhythmia, the different degrees of block in conduction, and the localized areas of rhythmicity. When these results are considered in conjunction with those on whole embryos taken at an early stage before nerve connections are established, they furnish additional support to the view that the depressing action of pressure on rhythmicity is not through an effect on the inhibitory nerve mechanism.

Discussion

In the agent pressure we have an instrument by which the contractility of cardiac and skeletal muscle tissue may be greatly stepped-up, but this action, remarkable as it is for these tissues, appears to be a peculiar effect on the contractile mechanism of these types of muscle. In the present experiments we have evidence that such fundamental biological properties as cell division and automaticity of the embryonic heart become restrained by pressure—a fact that presents equally difficult questions to answer as those given by the augmentation phenomenon in certain types of contractile tissue.

While the types of chemical change underlying the processes of cell division and heart rhythmicity obviously are complex and the factors common to both cannot be set down, yet the fact is not without interest that some forms of chemical reactions are known to be influenced by pressure. Rothmund (1896) found that the acid inversion of cane sugar, a first order catalytic reaction, is decreased in velocity about five per cent when subjected to a pressure of 500 atmospheres, and on theoretical grounds there is reason to conclude, according to Jones (1915), that the velocity of second order reactions is influenced in direct proportion to the pressure.

Attention was called in the early part of this paper to the observation that the cell constituents gave no gross signs of change under pressure.

With albumin, however, an addition of energy to the material produces a tendency to coagulation, as shown by Fermau and Pauli (1915) on irradiating with radium salts, and by Bridgman (1914) with high pressures; therefore it is probable that some alteration takes place in the egg substance even at the comparatively low pressures used in our experiments, but its character is such as to be not easily recognizable. The observations of Heilbrunn (1920, 1921) show in a definite way how great are the changes in viscosity of egg protoplasm during the process of division and they emphasize also the manner in which mitosis may be inhibited by factors that tend to modify the viscosity changes. The pressure effect in retarding cell division may be, therefore, largely one of altering the viscous properties of the cell constituents.

Perhaps the one factor upon which attention first becomes focused in the attempt to account for the decrease in division rate of eggs under pressure is a possible change in the oxygen supply. This feature received thoughtful attention at the outset of our experiments, and we believe that the details of procedure employed provide adequate controls and successfully rule out a change in gas tension as a factor in our results.

Our early observations on the effect of pressure gave evidence to support the view that the rhythmicity of the isolated heart is accelerated when a compression up to 60 atmospheres (882 pounds per square inch) is made to act upon this organ. The procedure in these initial experiments, however, of subjecting the preparation to pressure for only brief intervals and of taking records of the heart cycles immediately following the onset of pressure gave rise to an incomplete conception of the action of this agent. A review of our original tracings and evidence from many additional observations made since show quite conclusively that pressure does not induce an acceleration of the rate of the heart except as a temporary event. The present results, therefore, showing always depression of the rate under pressure seem at first discordant with previous findings, but full consideration of the facts indicates quite clearly that we are dealing with the secondary action of pressure on rhythmicity in contrast to the initial temporary effect that occurred during the first eight to ten cycles in a rhythmic preparation following the onset of compression.

The effect of pressure in slowing the heart rate raises a question concerning the mechanism of this action. While a mechanical factor may have contributed some part in this type of response, it is evident that such an effect does not arise from an increase in the resistance to the circulation by pressure narrowing the peripheral vessels, since careful observations of the size of certain small vascular channels reveal no detectable alterations in their caliber with the application of pressure. The property of automaticity of the heart, on the other hand, is influenced in a marked degree by changes in the ionic relations of its environment, and the observations of Bogojawlensky and Tammann (1898) on the conductivity-pressure coefficient of some conducting systems gives proof of the changes in the mobility of ions and of the alterations in the ionization of electrolytes by pressure. The precise changes in ionic equilibrium that may have operated in producing a retardation of the heart rate under compression cannot be given from the data at hand, but that a factor of this nature plays a part is strongly suggested by the type of effect that pressure gives in slowing the embryonic heart.

SUMMARY

Hydrostatic pressure of as much as 1900 pounds per square inch applied to living eggs of the *Fundulus* causes no evident changes in the cell constituents.

The rate of cell division of *Fundulus* eggs was slowed by maintaining them under pressures of 1300 to 1900 pounds for periods ranging from 30 minutes to three hours. In the pressure samples a few instances appeared of abnormal forms of development.

The automaticity of the heart in young embryos becomes slowed by pressure and apparently may be abolished if the compression is maintained but returns within a few minutes following release of the pressure. In ten experiments an average decrease of 9.9 per cent in the heart rhythm under pressure was present within an interval of two minutes. The embryonic heart under pressure develops arrhythmia, types of local block, and isolated fibrillary activity.

The action of pressure in slowing the heart rate does not depend on the presence of inhibitory nerves to this organ, since compression produces a slower rhythm of (*a*) hearts from older embryos that have been dissected free of all extrinsic nerve connections; and (*b*) hearts of young embryos that have no intrinsic nerves developed.

The bearing of viscosity and ionic changes produced by pressure are considered in relation to the decrease in the rate of cell division and the automaticity of the embryonic heart.

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THE CRUSTACEAN EYE HORMONE AS A VERTEBRATE MELANOPHORE ACTIVATOR

EARLE B. PERKINS AND BENJAMIN KROPP

ZOÖLOGICAL LABORATORY, RUTGERS UNIVERSITY

There is produced in the eye stalks of crustaceans a hormone which, carried in the blood stream, is effective in inducing chromatophore changes. This has been demonstrated repeatedly by Perkins (1928) and Perkins and Snook (1931) for *Palæmonetes*, and by Koller (1928) for *Crangon*. The active material acts in every case as a contractor,—that is, an extract from eye stalks, when introduced into the circulation of blinded or black-adapted shrimps, causes maximal contraction of the red and yellow chromatophores. Koller (1928) also reported the occurrence of the reciprocal phenomenon, namely, expansion of chromatophores of white-adapted shrimps induced by extracts from the rostral region of *Crangon*. Thus far this has failed of experimental confirmation (Perkins and Snook, 1931).

In 1929, Kropp reported the presence in the eyes of black-adapted tadpoles of a substance effective in producing expansion of melanophores in white-adapted *Fundulus* and tadpoles of *Rana clamitans*. In this case the use of extracts from eyes of white-adapted tadpoles gave no results on black-adapted animals. Here, as in the case of the invertebrates, evidence pointed to the presence in the eyes of a substance which, under proper conditions, induced effects on chromatophores.

To test the interspecificity of the chromatophore activator found in the crustacean eye stalk, and thus further to establish its hormone nature, was the object of the experiments here reported. Extracts from the eye stalks of *Palæmonetes vulgaris* were made in 0.7 per cent NaCl solution. Thirty eye stalks were macerated in 5 cc. salt solution, boiled, centrifuged, and the clear extract decanted off and cooled. Tadpoles of *Rana clamitans* were placed in white and black dishes until their skin melanophores were respectively nearly maximally contracted and expanded. To the unaided eye the white-adapted tadpoles appeared light yellow-green, the black-adapted almost black. Each individual then received 0.2 cc. of the extract in the dorsal lymph sinus. White-adapted controls received 0.2 cc. 0.7 per cent NaCl solution. Animals adapted to a black background, which received 0.2 cc. of the eye extract, showed no obvious melanophore movements as a result of the injection, nor did the controls at any time show marked changes in pigmentation. The animals adapted to a white background, however, did show pronounced effects. Within five minutes of the injection these tadpoles began to darken over most of the dorsal surface, even though they remained on a white background. Maximal darkening was reached after thirteen minutes. The tadpoles at this time showed a very dark band across the whole dorsal surface, extending forward to a line connecting the posterior margins of the eyes, and backwards to the anterior root of the tail (Fig. 1). The snout region remained characteristically in the original light condition when the injection was made into the dorsal lymph spaces. We assumed that since the skin between the eyes and over most of the head adheres closely to the cranium, the lack of response of the

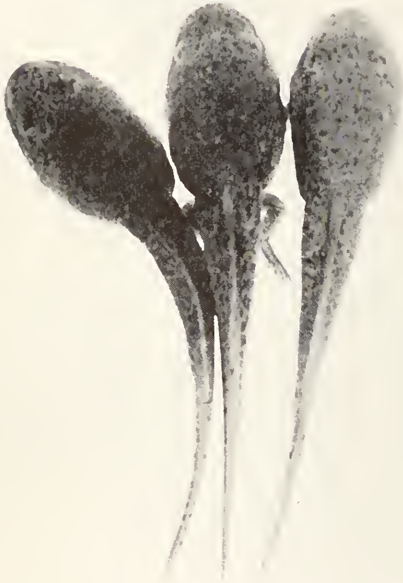

FIG. 1. Left, black-adapted tadpole injected with 0.2 cc. *Palæmonetes* eye extract. Center, white-adapted tadpole injected with 0.2 cc. *Palæmonetes* eye extract: melanophores expanded following injection. Right, white-adapted tadpole injected with 0.2 cc. 0.7 per cent NaCl: no expansion of melanophores.

head melanophores was due solely to the presence of this mechanical barrier and the consequent inability of the material to come into contact with the skin in this region. This assumption proved correct, for injection of the extract directly under the head skin produced the darkening reaction over the head. The tail did not show the effect, although a suggestion of it could be obtained by injecting the extract directly into the tail musculature. This is only an apparent anomaly and by no means real, as will be shown below.

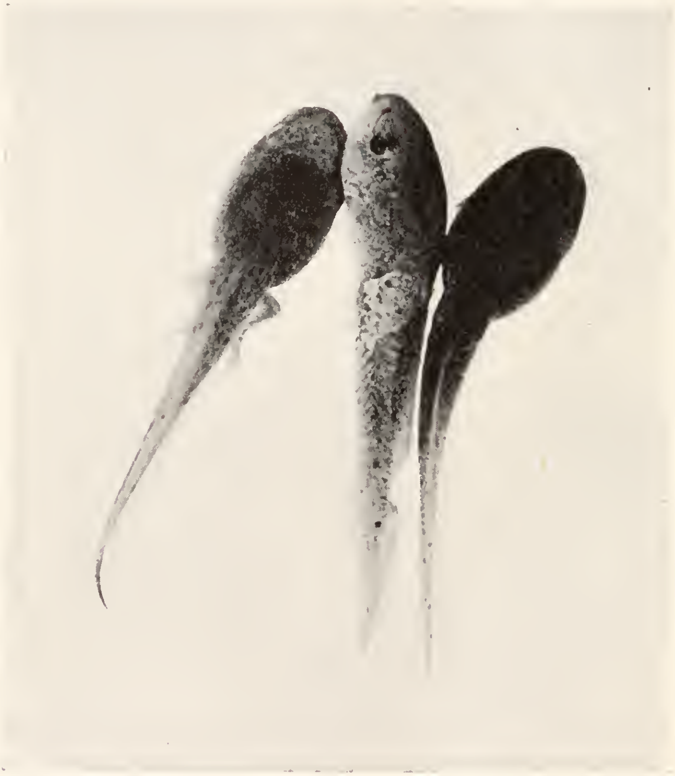

FIG. 2. Left, eye-extract injected white-adapted tadpole. Center, pallor appearing after effect of injecting eye extract had worn off. Sharp line of demarcation between area of punctate melanophores and stellate melanophores of tail clearly shown. Right, normal black-adapted tadpole.

Microscopic examination of the skin of the living animal at this time revealed the expected condition—a dense network of expanded melanophores all over the darkened area. Fifteen minutes after the injection the darkening began to disappear until, thirty minutes after maximum darkening, the animal was once more in its original light state, the effects of the extract apparently having been dissipated. The process of returning pallor did not, however, stop at this point. It continued until the entire dorsal surface of the animal became lighter than it was before the extract was injected. This after-pallor may persist for twenty-four hours or more. It is confined to the dorsum and the root of the tail, the greater mass of the tail being non-reactive and appearing dark in contrast to the pale dorsal surface (Fig. 2). Microscopic examination of the living animal's skin showed, as expected, sharply contracted, punctate dermal melanophores in the trunk, while those of the tail were stellate. The region of demarcation between trunk and tail was strikingly sharp and in different animals was surprisingly similar in contour. The darker area of the tail formed a **V**, pointing cephalad, at a point just posterior to the union of tail and trunk. Laterally the line ran posteriorly and ventrally until near the ventral raphe of the tail, where it continued laterally but anteriorly and was lost in the lightly pigmented skin of the venter. In some animals the line followed superficially the course of the spinal nerves in the tail, while in others there was a more irregular course.

In addition to eye stalks of *Palæmonetes vulgaris* we used those of the blue crab, *Callinectes sapidus*. The active substance was present here also, and the results described above hold true in every detail for tadpoles treated with extracts of eye stalks of *Callinectes*. The only differences observed were that with *Palæmonetes* extracts the onset of the darkening reaction was more rapid, and the degree of maximum darkening attained was somewhat greater.

Since hormones are carried by the blood stream to all parts of the organism, it appears irregular at first sight that the tadpole tail should not respond as did the dorsal surface of the trunk. The tadpole tail has often been an object for experimentation on color change and pigmentary reactions, but results have usually been inconclusive or contradictory (Kropp, 1927). It is indeed not strange that this is so, for the tail of the tadpole, especially in older specimens and when the hind legs have appeared, is in a state of physiological flux. The disorganization of the pigmentary system may be correlated with circulatory changes and the general resorption process of this organ. The fact that the tail is a poorly reacting system is probably due to this physiological instability. In our experiments the reacting substance was carried by lymph and to some extent by the blood stream. For practical reasons we did not inject intravenously at this time. However, we venture the prediction that intravenous injection will not greatly alter the reaction of the tail as described above.

The injected substance has opposite effects in the shrimp and in tadpoles,—producing contraction of chromatophores in the former, expansion in the latter. This is a further example of a hormone producing different results depending on the reacting system or type of chromatophore concerned.

The chromatophore activator produced by the crustacean eye stalk shows all the characteristics of a true hormone now that we may include its wide interspecificity. This has recently been reported also by Koller and Meyer (1930) and by Meyer (1931), who injected eye extracts and rostral-region extracts of *Crangon* into the fishes *Gobius* and *Pleuronectes,* producing chromatophore movements. With the eye-stalk extract they report the production of pallor in these fishes,—the opposite of the effect we obtained on frog tadpoles.

The fact that an invertebrate hormone is effective on a vertebrate system seems highly significant. As yet very few endocrine processes are known among the invertebrates—the crustacean eye hormone being by far the most spectacular. The attention that has been given to the vertebrate endocrine system has disclosed a variety of humoral effects, but it is by no means certain that this important coördinating mechanism is confined to the vertebrates alone. It is possible that every tissue possesses the ability to affect other tissues by means of substances liberated into the circulating media, not only in the vertebrates but in the invertebrates as well. The occurrence of such a substance in crustaceans which is effective in fishes and amphibians may indicate an evolutionary precursor of the more highly developed vertebrate endocrine system.

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"MITOGENETIC RAYS"—A CRITIQUE OF THE YEAST-DETECTOR METHOD

OSCAR W. RICHARDS AND G. WELLFORD TAYLOR[1]

(From the Osborn Zoölogical Laboratory, Yale University, the Physiological Laboratory, Princeton University, and the Marine Biological Laboratory, Woods Hole)

The interest in the theory of mitogenetic radiation has brought forth a considerable literature. Schrieber and Luntz (1931) recently list 49 cases indicating the existence of these rays and 26 cases where negative results were obtained when yeast was used as a detector for the radiation. These rays of short wave length (2000–3400 Å) are supposed to be given off by a wide variety of tissues and to possess the property of accelerating the division of other cells placed in their path. Further, the budding of yeast is generally considered by the proponents of this theory to be especially sensitive to these rays and to be one of the best detectors of this radiation. The literature has been reviewed recently by Hollaender and Schoeffel (1931) and by Taylor and Harvey (1931). Baron (1930) reports mutuoinduction or the acceleration of the budding of yeast by their own radiation.

I

The husbandry of the yeast in many of the published papers on the effects of such radiation is not satisfactory to one familiar with the growth of yeast, and the following minimal essentials are stated to establish criteria for the evaluation of experimental data on the theory of mitogenetic radiation. Violation of these well-established fundamentals of yeast culture accounts for much of the conflicting opinion and inadequate literature on this subject.

First, the yeast used as a detector for this radiation must be a pure strain obtained from an isolated, single cell. Single cell isolation studies by Wallace and Tanner (1928) have shown the amount of variation of different cells from the same pure species of yeast. Second, both the rate of the growth and the yield of yeast should be reported as well as the percentage of cells with buds. An increase in the population, over a period of time, is a better index of proliferation than the percentage of buds. Third, the mortality of cells must be determined at the same time. A high mortality would vitiate such experiments and evidence should be produced that there has been no selective killing as normally occurs during the latter part of the growth of a population when the culture medium is not maintained effectively constant (Richards, 1928*a*, *b*, 1932*a*). Fourth, the relative and absolute errors of sampling the populations and of the counting, etc., must be measured and stated. Fifth, the yeast should be maintained at the proper temperature for the species studied and the temperature used stated (*e.g.*, not just as room temperature, Streline, 1929). *Saccharomyces cerevisiæ* shows irregularity of division with elongate cells at 30.0° and injury at higher temperatures.[2] Sixth, the medium should be maintained effectively constant, except for the radiation of the experimental cultures, so that the rate of growth of the yeast otherwise is constant. With many of the culture fluids this limits the length of the experiment with respect to the amount of seeding and the volume of the medium to less than 40 hours unless the medium is changed during the experiment. For a given amount of medium a definitely limited yeast crop will be obtained when the food is exhausted and the waste products accumulate in sufficient concentration to check the growth. With large seedings this crop is obtained in less time than with smaller seedings, even though the rate of growth (percentage and rate of budding) may be the same in both cases. When very small amounts of culture medium, single drops, are used the succession of the known changes which determine the growth of the population [3] would influence and obscure the effects of radiation. This is especially true with experiments on mutuoinduction when the cell densities are proportionally greater.

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[1] Mr. Taylor expresses his appreciation to the staff of the Woods Hole Laboratory of the U. S. Bureau of Fisheries for facilities placed at his disposal during the summer of 1931 when these experiments were performed.

The proponents of the theory of mitogenetic rays have implied that yeast is unsatisfactory as a detector when it is growing at a constant rate in an effectively constant environment because it is then growing as rapidly as possible. They presume that there is a latent period in cell division which limits the rate of its recurrence. This objection to constant conditions does not affect the experiments to be described because with the same culture conditions the addition of a growth stimulant, such as inosite (Richards, 1932*b*), in a concentration of 1 : 100,000 will significantly increase the rate of growth. It is also to be remembered that the budding of most of the common species of yeast is different from that of cultures of tissues from multicellular organisms in that the same mother cell may form simultaneously two or more buds. Baron

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[2] Borodin (1930) neglects this fact, which probably accounts for much of the variation in his experiments (*cf.* Richards, 1928*c*).

[3] *Cf.* Richards (1932*a*) for measurements and references to the yeast literature.

(1930) states that the initial inoculation must be less than 7,000 per cc. because when a greater concentration is present the cells are saturated with their own radiation and insensitive to radiation from another source. The experiments herein reported had initial concentrations of about 1,200 cells per cubic centimeter.

The observation of the mass of yeast in a hanging drop is not an accurate method, since the cells settle to the bottom of the drop and more cells are present below the center of the drop than at the sides. This error was very obvious in the older type, now generally abandoned, of hæmocytometer made with a center disc surrounded by a circular moat. Even with the use of a planimeter as suggested by Borodin (1931) it is doubtful whether accurate determinations could be made, for the above error of curvature is not avoided and it is exceedingly difficult to estimate the number of cells present from small differences in the apparent optical density of the settled yeast. Counting the cells, the method used in our experiments, not only avoids these special difficulties but also makes possible the estimation of the variation of the measurements, which is important in establishing the accuracy of the method. This is especially important when the method is used to demonstrate a hitherto unknown phenomenon of nature.

II

In the first experiments reported here the yeast was grown in glass or quartz test tubes containing 1.5 to 2.0 cc. of sterile medium and the tubes arranged as shown in Figure 1. The tubes were immersed in a dish containing the bacterial suspension. Sufficient air was drawn through the suspension of bacteria to insure an adequate supply of oxygen. The glass tubes were of ordinary lime glass. The quartz tubes were known to transmit ultra-violet light of the wave length assigned to the mitogenetic rays. This volume of medium was desirable in order that the errors of sampling the yeast population for the counts should be reduced to a minimum. At each time recorded in the tables the cells were shaken to give a uniform suspension and then a small amount of the suspension was transferred to a hæmocytometer and the number of cells present in 5 to 20 unit volumes of 1/250 cu. mm. was counted, using the technique developed by Richards (1928*a*, *d*, 1932*a*). The number of buds present was counted and at certain intervals the number of injured or dead cells was determined. No significant differences in mortality were found between control and experimental cultures, and as the mortality during the period of logarithmic growth (rate of growth constant) used in these experiments is slight, this information is not reported here. The criterion of a bud was arbitrary in that all buds that were seen formed but not yet separated by a wall from the mother cell were counted. Since the senior author made all of the counts of the yeast, the figures are directly comparable. The bacterial suspensions were maintained in a nutrient medium in which they continued to grow over a period of at least eighteen hours. In the longer experiments the bacteria were changed at intervals to insure that a constantly fresh and actively growing suspension was present during the entire course of the experiment. The bacterial cultures were cared for by the junior author. The cultures were maintained in an incubator at 27.8 ± 0.2° C. A special control for each experiment was accomplished by having a glass and a quartz test tube (referred to in the tables as G_0 and Q_0 respectively) at the same temperature as the other cultures but shielded by glass from the hypothetical source of the mitogenetic rays.

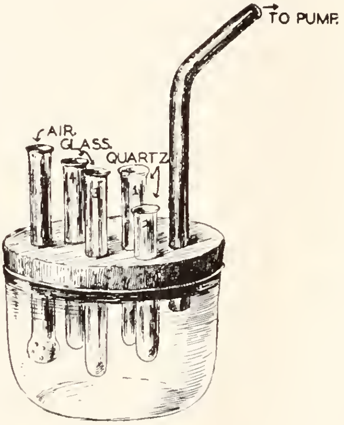

FIGURE 1.

Fig. 1. Arrangement of tubes for Series 109–113. Cotton plugs of tubes, yeast, and bacterial suspensions not shown.

Mitogenetic rays are supposedly effective in accelerating cell division for a distance of several centimeters. The tubes used in these experiments, about one centimeter in diameter, were small enough to insure exposure of all of the cells to any radiation produced by the several sources used. The growth of the yeast in the volumes used was more uniform than usually occurs on agar slants, which generally have been used in the experiments by other investigators. As will be shown later, the actual counts of the population density make certain that there has not been any small increase in the number of cells, that might be missed in counts of the percentage of buds in smears made from the growth on an agar surface. This is important as these initial small differences would become more divergent as the logarithmic increase continued.

The first two sets of experiments were made with William's medium, a pure strain of *Saccharomyces cerevisiæ* Hansen as the detector, and a

TABLE I

Percentage of Budding and "Induction"

Series No.	Time in hours	PERCENTAGE OF BUDDING							PERCENTAGE OF "INDUCTION" WITH BACTERIA				
		No bacteria (special control)		With bacteria				No bacteria (special control)					
		Quartz tube	Glass tube	Quartz tubes		Glass tubes							
		Q_0	G_0	Q_1	Q_2	G_3	G_4	$\frac{Q_0}{G_0}$	$\frac{Q_1Q_2^*}{G_3G_4}$	$\frac{Q_1}{G_3}$	$\frac{Q_2}{G_3}$	$\frac{Q_1}{G_4}$	$\frac{Q_2}{G_4}$
Series 109	2	18	26	20	17	17	27	−31	−16	+18	± 0	−26	− 4
	4	24	28	29	14	20	16	−14	+19	+45	−30	−80	− 1
	6	19	14	25	14	20	20	+36	− 2	+25	−30	+25	−30
	8	14	14	21	23	15	17	± 0	+25	+40	+53	+24	+35
	22	13	12	13	13	12	17	+ 9	−10	+ 8	+ 8	−24	−24
Series 110	2	19	18.5	18.5	17	20	20	+ 5	−16	−11	−22	−11	−22
	4	18.5	20.5	17.5	16	16.5	22	− 1	−12	+ 6	+ 3	−11	−28
	6	16.5	12.5	12.5	15	14	26	+36	−31	−11	+ 7	−51	−44
	9	11.5	13	13.0	16.5	13	16.5	−12	± 0	± 0	+19	−15	± 0
	24	7	10.5	12.5	13.5	14	9	−33	+13	−11	− 4	+39	+50
Series 112	3	28	27	35.5	32	34	28.5	+ 3	± 0	+ 4	− 6	+25	+12
	5	21	26	20.5	16	18	23	−19	−15	+14	−11	−11	−30
	8	18	22	21.5	17	23	21	−18	− 6	− 7	−26	+ 2	−19
	12	25.5	30.5	35.5	37	38.5	33	−16	+ 1	− 8	− 4	+ 8	+12
	24	23.5	24	21.5	20.5	20	25.5	− 2	− 8	+ 8	+ 3	−16	−20
Series 113	3	35	29	30.5	29.5	34	26	+27	± 0	−10	−14	+40	+12
	7	22	27	25	23	25	23	−23	± 0	± 0	− 8	+ 9	± 0
	11	24.5	26.5	22	18.5	23	24.5	− 8	−16	− 4	−20	−10	−25
	24	28.5	28.5	39	42	39.5	42.5	± 0	− 1	−13	+ 6	− 8	− 1

* Calculated from the average percentage of budding with the two quartz tube and the two glass tube populations. Q_1G_3 was calculated from the first quartz tube percentage and from the first glass tube percentage, etc., for all four combinations of the data.

culture of luminous bacteria, from a single cell of *Vibrio phosphorescens,* for the generation of mitogenetic rays. The results are reported in Table I, Series 109 and 110, The percentage of induction, I, was calculated from the formula given by Gurwitsch (1925):

$$I = \frac{Q - G}{G},$$

where Q is the percentage of budding in the quartz tubes and G the percentage of budding in the control or glass tubes. In the special control of a glass and a quartz tube shielded from any rays an apparent positive induction occurred four times out of ten possibilities (Table I, columns Q_0 and G_0), two of which were within the significant range, according to Gurwitsch (1925), of 30–120 per cent even though *no bacteria* were available to generate mitogenetic rays. The rest of the observations were negative or zero. When the percentages of the two quartz and two glass tubes placed in the bacterial suspension were averaged respectively and the averages used to calculate the induction (columns Q_1 Q_2, G_3 G_4), only three positive cases in ten experiments were found, and in none of these was the induction great enough to be significant.

It is further desirable to calculate the induction from the four combinations of the percentages of the first glass control tube population (G_3) and the first and second quartz (Q_1 and Q_2) tubes separately and likewise with the second glass control tube (G_4) and the two quartz tube percentages of budding. Examination of the results tabulated in the last four columns of Table I shows only 15 positive cases of the 40 computed percentages and of these only 7 are of sufficient magnitude to be significant even though all had been exposed to an actively growing culture of bacteria which was present to generate mitogenetic radiation.

Another species of yeast, *Saccharomyces ellipsoideus,* has been suggested as being possibly more sensitive to mitogenetic rays than *S. cerevisiæ,* so we obtained a transfer of culture No. 4116 from the American Type Culture Collection and repeated the above experiment using this species. This time we used malt extract medium, which is a very suitable culture fluid for this species, and the results of this experiment are given as Series 112 in Table I. Distinctly less induction was observed with a preponderance of negative cases. It is noteworthy that one significant positive case of 36 per cent was obtained in the special control without radiation, since this shows the amount of variation occurring with very carefully controlled conditions.

The only explanation for these consistent negative results is the possibility that luminous bacteria, or at least the species here used, do not emit mitogenetic radiation. In order to show that this bacterium is not unique in this respect, the above experiments were repeated using *Phytomonas tumefaciens* (Am. Type Coll. No. 4452), as this species has been reported by other experimenters to be a good generator of mitogenetic rays. The observations are recorded in Table I as Series 113 and only one significant positive case of induction was observed out of eight observations.

During most of the period of the experiments the growth of the yeast is logarithmic. Should there be any acceleration of the multiplication of the yeast due to stimulation by mitogenetic radiation, it should show not only as an increase in the rate of growth but also as an increased yield in the quartz tubes over that obtained in the glass tubes. A slight acceleration, possibly less than would be noticed in the percentages of budding, would be distinctly noticeable by the end of the period of growth. To test this the ratios of the number of cells in the quartz tubes to the number in the glass tubes at the end of each series were calculated. For the control which was shielded from the bacteria the average of the ratios was 0.91, showing that there were slightly more cells present in the glass than in the quartz tubes. The average of the same ratios for the tubes suspended in the bacterial culture was 0.99. Since twice as many counts were averaged than with the special control, more of the variation is cancelled, which accounts for the index being nearer unity. The maximum yield is less in the quartz tubes than in the glass tubes, which again fails to indicate any stimulation of budding attributable to a mitogenetic radiation.

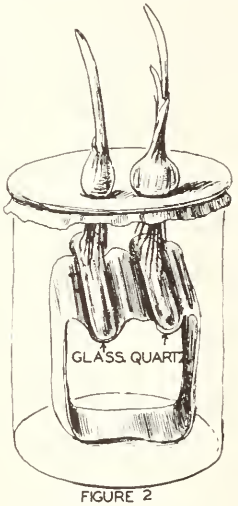

FIGURE 2

Fig. 2. Arrangement for exposing tubes to radiation from onion roots. When in use, the beaker was partly filled with water to prevent drying of the onion roots.

When the logarithm of the number of cells is plotted against time, the slope of the resulting curve is the relative rate of multiplication of the cells. This graph is linear during all of these experiments except the last observation of five cultures, because the seeding was from actively growing cultures. These curves were plotted for each tube of the four series and the slope of the curve measured with a tangentmeter (Richards and Roope, 1930). An index was obtained by dividing the tangent of the curve from a quartz tube by that from the corresponding glass tube. The average of these ratios for the control with no bacteria was 0.97 and for the tubes in the bacterial suspension 0.86. This shows that the rate of growth was slightly greater for the yeast populations in glass tubes, which might be due to some material in the glass dissolving into the medium, which causes a very slight increase of growth. This could not be enough to impair the value of the glass as a control as the differences are but slightly more than the probable error of the differ-

TABLE II

Percentage of Budding and "Induction"

Series	Time	Budding in Quartz	Budding in Glass	Induction
	hours	*per cent*	*per cent*	*per cent*
117	2	21	24.5	−14
	4	23	28	−18
	6	27.5	28	− 2
	8	33.5	33	+ 2
	11	22.5	26.5	−15
	23	23	23	− 8
118	2	22	33	−33
	4¼	28.5	32.5	−13
	8¼	35	30.5	+15
	12¼	36.5	31.5	+16
	26	27	28	− 4
119	2	37	34.5	+ 7
	5	37	38.5	− 4
	7	35	45	−22
	12.5	35	40	−12.5
	24	37.5	35.5	+ 4
				Summary 5+ 31% 11− 69%

Ratios Quartz/Glass

	Series	Growth rate	Yield	Detector	Generator
	117	1.07	1.11	*S. ellip.*	Onion
	118	.96	1.05	*S. cerev.*	Onion
	119	1.00	.84	*S. ellip.*	Onion

ences, and the sign of the difference was not the same in the several experiments. This is not due to inhibition, because some of the experiments gave greater growth in quartz than in glass. These two additional criteria also indicate that there is no influence passing through quartz but not through glass to stimulate the multiplication of yeast.

Three series of experiments were made using onion roots as the generator of mitogenetic rays and using both species of yeast as detectors. The arrangement of the experiments is shown in Figure 2. Since the yeast suspension in the tubes covered a considerable area, all of the roots were left on the onion and the positions of the tubes were reversed at each count to insure comparable conditions. The results are given in Table II. The negative cases again predominate and no essential differences are noted in the ratios of the rates of growth or in the yield between the experimental and the control cultures.

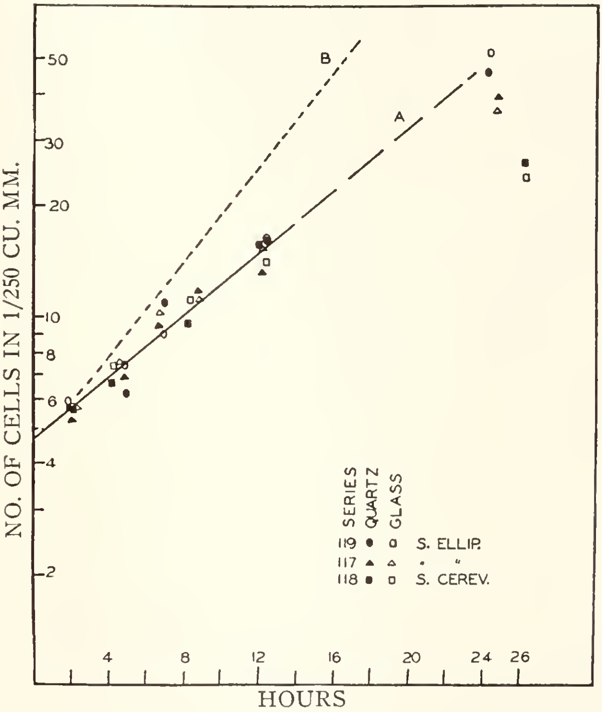

Fig. 3. *A*. The growth curve of Series 117–119. *B*. Hypothetical growth curve that would be obtained with a 10 per cent stimulation of cell multiplication (*cf.* text).

The actual counts of the growth of cells are given in Fig. 3, curve *A*. Each point is an average of several counts for a single tube. Because only one tube was used in each experiment, all of the variation is shown in the figure. The growth for the first twelve hours is logarithmic, but the final observation of both Series 117 and 118 was beyond the period of constant growth rate. The effects of a lag period are slight. Careful examination of the points shows no consistent change in the growth that can be interpreted as an acceleration or an acceleration followed by a retardation. The average growth of the onion roots in the moist atmosphere during the experiments was 15 per cent.

The concluding experiments were designed to test whether yeast would stimulate other yeast when the two populations were separated by quartz. The arrangement of the dishes is shown in Figure 4. Two cubic centimeters of the yeast suspension were used for both the generator and detector populations. This amount filled the space in the lower container up to the quartz partition and is about the smallest amount that allows counts without considerable errors in sampling. The observations are given in Table III. Two duplicate experiments were made in each case. The induction percentages for the averages of both duplicates give an equal number of positive and negative cases in none of which are the differences great enough to be significant. When all combinations of the experiments are calculated, there is again an excess of negative cases. The average figures suggest that there may be some increased budding in the quartz dishes at two to four hours after seeding. These differences are not of sufficient magnitude to be significant and in the case of Series 119, the positive deviation is due to variation in only one of the two populations.

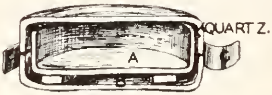

FIGURE 4

FIG. 4. Cross-section showing arrangement of a quartz dish within a Petri dish. The detector yeast was placed at *A* and the generator yeast at *B*.

III

There is noticed a considerable variation between the different cultures even though conditions were made as nearly uniform as possible. The extent of this natural variation was measured in several cases, two of which are given in Table IV showing the percentages of buds counted and the probable error of the percentages. This was obtained by keeping a separate record of the counts in each of the 20 unit volumes counted and the usual statistical formula applied. With divergent percentages (Case 1) the induction varies from 2 per cent to 35 per cent for values within the range (mean ± its probable error) wherein the occurrence of another observation is expected to be equally probable. This result is important, as percentages of induction greater than 30 per cent are significant according to Gurwitsch (1925). Where the values approach each other more closely (Case 2), the induction calculated varies from — 2 per cent to + 33 per cent; or from a negative

Table III

Percentage of "Induction"

Series	Time	Percentage of induction Av. Glass / Av. Quartz	Q_1G_1	Q_1G_2	Q_2G_1	Q_2G_2
	hrs.					
117a	2	+ 2	± 0	+ 4	± 0	+14
	4	+27	+39	+45	+ 9	+14
	6	± 0	−13	+11	− 6	+20
	8	−26	−26	−26	−26	−26
	11	−17	−11	−14	−16	−19
	23	−20	−15	−19	−19	−27
118a	2	−12	−21	−11	−13	− 2
	4	+28	+25	+21	+27	+33
	8	−13	− 6	−17	− 7	−18
	12	+ 3	−19	−14	+19	+28
	26	+11	+ 7	+ 7	+15	+15
119a	2	− 4	+27	− 1	− 4	− 8
	5	+14	−21	−10	− 4	+ 8
	7	−12	− 1	− 7	− 9	−14
	12.5	+12	+15	+30	−11	+ 2
	24	− 2	−11	− 7	+ 4	+ 9
		Summary 8+ 50% 8− 50%		Summary 25+ 38% 2±0 4% 37− 58%		

Initial concentration of yeast (cells in 1/250 $mm.^3$)

Series	Generator	Detector
117a	70.8 *S. ellipsoideus*	11.0 *S. ellipsoideus*
118a	47.1 *S. cerevisiae*	6.4 *S. cerevisiae*
119a	39.0 *S. cerevisiae*	5.1 *S. ellipsoideus*

result (less budding in quartz than in glass) to a significantly positive result. This clearly shows that the index of induction obtained from this formula is invalidated by the *normal variation* of the biological material and is, therefore, *not a satisfactory criterion* for establishing the existence of mitogenetic radiation.

Gurwitsch (1931) has recently indicated that continued radiation may produce an inhibitory effect on cell multiplication. The above experiments do not support this hypothesis because in them the direction of variation is independent of time. Further, it would be improbable that the inhibition would be such as to give, in all cases, the same or slightly less variation than the amount of control growth when the concentrations of the generator and detector populations varied over the limits used in our experiments (*cf.* Table III).

Table IV

Effects of Variation on "Induction" *

	PERCENTAGE OF BUDS COUNTED		PERCENTAGE OF "INDUCTION"
	Quartz tubes	Glass tubes	
Case 1	30.5 ± 2.1	26.0 ± 1.8	
	30.5	26.0	+17
	30.5 + 2.1 = 32.6	26 − 1.8 = 24.2	+35
	30.5 − 2.1 = 28.4	26 + 1.8 = 27.8	+ 2
	32.6	27.8	+17
	28.4	24.2	+17
Case 2	35.9 ± 2.4	31.5 ± 2.7	
	35.9	31.5	+14
	38.3	28.8	+33
	33.5	34.2	− 2
	38.3	34.2	+12
	33.5	28.8	+16

* In each case the mean and its probable error are given. The "induction" is calculated with the Gurwitsch formula (*cf.* text) from the mean values and for the combinations of the mean values both plus and minus the probable errors of the respective means.

Baron (1930) reports mutuoinduction in yeast populations when he used hanging drops with different densities of cells and rounded up or spread out in form. He starts with cells having no buds and reports the percentage of cells which still have no buds at each observation. The difference in the percentages indicated increased budding when the cells were in close proximity, which increase passed through a maximum and then decreased. A few negative cases were found and explained by having too densely seeded original populations. Baron attributes the shortening of the lag period to premature stimulation of budding by the radiation of the cells on themselves, or by the radiation of other cells, depending on the arrangement of the experiments.

Three other explanations are possible besides the one that he gives. Eijkman (1912) has shown that the order of spore germination is logarithmic. The recovery from previously unfavorable environmental conditions which stopped budding is also probably logarithmic, so that the numbers would pass through a maximum as the logarithmic order was decreased by the increasingly unfavorable conditions of the small, single drop environments. Another possibility is that the presence of more cells may reduce the lag period by some chemical effect of the aggregation, of the nature discussed by Allee (1930). This is less likely, since Peskett (1927) was unable to demonstrate any allelocatalytic effects on the growth of yeast. Possible effects of a chemical conditioning of the medium should be tested for when large concentrations of cells are present, before a decreased lag period can be attributed to the effect of autoradiation.

Baron did not report any counts of the cells present so a more complete analysis of this experiment is not possible, nor is it clear that he worked with homogeneous material derived from a single cell isolation. It is possible that his denser suspensions used up the food in the drops more rapidly and that and the resulting increase of toxic waste products brought about the diminution of budding which he observed. Slator (1921) has stated that a considerable concentration of cells will fail sometimes to come out of the lag period and increase their number. A combination of these effects could give Baron's results, and it is believed his observations can be accepted only when he measures the changes in the environments to demonstrate that the environmental changes have no effect that might be attributed to radiation, or better, maintains the environments effectively constant except for the radiation of the experimental cultures. The changes in the environment as cells grow when the medium is not renewed have been measured for larger environments, but not as yet for the smaller environments. When the environment is restricted to a single drop, these changes must regulate the growth of the yeast more effectively than with larger environments. It is probable that failure to avoid the effects of such changes accounts for the difficulties that have prevented direct comparison of experimental findings with hanging drop and agar block cultures.

Clark (1922) varied the seeding from five cells to eight million cells per cubic centimeter with a strain of *S. cerevisiæ* different from that used in our experiments and found no significant differences in the rate of growth of his populations that could be attributed to a mutuoinduction of the cells upon each other. Richards (1932*a*) found that the rate of growth, with the same strain of yeast, was the same when the initial seeding was varied from fifteen thousand to one and one-half million yeast cells per cubic centimeter of medium.

Streline (1929) finds an initial stimulation of growth due to mitogenetic radiation which rapidly disappears. He emphasizes the need for counts of the number of cells present and for liquid culture medium. Streline uses *Nadsonia fulvescens*, which has a different form of budding from the yeast reported in this paper, and in reporting his experiments counts two small buds as one cell. This prevents further analysis of his data, and without knowledge of the mortality of his cells it is not possible to analyze the interesting disappearance of the stimulation. He, too, reports some negative cases.

IV

When there is no killing of the cells in the population, any stimulation of cell proliferation would give greater differences between the size of the control and the experimental populations. For instance, had there been a 10 per cent increase of cells in the experiments reported in Fig. 3, curve *A*, during the first two hours that had continued, the increase that would have occurred is shown by the broken line, Fig. 3, curve *B*. Had there been an initial stimulation that had not continued, then the growth curve would have been a line above and parallel to the growth curve of the control. The figure demonstrates that no such stimulation occurred in our cultures.

In our experiments the environment was maintained effectively constant, except for the possible radiation of the experimental cultures, and the actual variation in both the number of cells present and the number of buds and in the death rate was measured. From these data the rate of growth and the percentage of budding were calculated.

These carefully controlled experiments, using *S. cerevisiæ* and *ellipsoideus* for detectors, and onion root, two species of bacteria—*V. phosphorescens* and *P. tumefaciens*—and the two above-mentioned yeast as generators, show no stimulation of the multiplication of yeast by mitogenetic radiation that might pass through quartz but not through glass.

It is not possible to compare our experiments closely with those obtained by other investigators because the latter have not given their complete data. Only when the complete evidence, as has been indicated in Sections I and III of this paper, is given together with a definite acceleration of the multiplication of yeast by mitogenetic radiation, can a theory of mitogenetic radiation be established. The formula for the percentage of induction proposed by Gurwitsch is inadequate and unsatisfactory because it too greatly exaggerates the normal variation of the growth of yeast.[4]

Summary

Yeasts (*Saccharomyces cerevisiæ* and *ellipsoideus*) grown in a liquid medium which was maintained effectively constant in quartz and in glass containers, were exposed to supposedly potent sources of mitogenetic radiation: bacteria (*Vibrio phosphorescens* and *Phytomonas tumefaciens*), onion roots, and the two above-mentioned species of yeast. In 58 per cent of the experiments, negative results were obtained, while in 42 per cent there was a slight positive variation which did not exceed normal control variations. No effects of a mitogenetic radiation could be detected in the exposed yeast. In all cases the percentage range of variation of the experimental cultures was within the range of normal variation.

It is further shown that the formula used by the Gurwitsch school in calculating the induction effect is unsatisfactory because it exaggerates the variations found in the normal cultures until they appear as induction or inhibition effects.

The necessity of an effectively constant environment which does not restrict the potentially unlimited growth of the yeast is stressed. A constant environment avoids the difficulties of measurement and evaluation of the unfavorable effects of changing environments in experiments designed to test the effect of mitogenetic radiation or other stimulants of yeast growth.

Thus certain conditions and criteria are established which, it is believed, the proponents of the theory of mitogenetic radiation cannot legitimately disregard in their attempt to establish the existence of such a radiation when yeast cultures are used as detectors.

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[4] If the conclusions of Rahn (1932) are correct, this normal variation never can be avoided completely.

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RATE OF REGENERATION OF PARTLY HISTOLYZED ANURAN TAIL SKIN[1]

H. J. CLAUSEN

Department of Biology, University College, New York University

Introduction

Recent studies on anuran integument and muscle transplantations (Helff, 1926; Lindeman, 1929*a*; Helff and Clausen, 1929; and Clausen, 1930) all suggest the probability that the causal histolytic influence of the various tissues of the tail may be due to specific substances in the blood stream or a general lowering of the pH of the blood during metamorphosis. It has been suggested (Lindeman, 1929*b*) that this influence is essential, not only as an initiatory agent, but must also be present continuously during tail resorption. This was demonstrated by the transplantation of histolyzing anuran tail-skin to normal individuals, resulting in the normal reconstitution of the graft in question. The various intermediate regenerative stages were not described, however. Part of the present paper is devoted, therefore, to a description of the normal histological regenerative processes, which occur in tail integument following partial histolysis.

In a previous paper (Clausen, 1930) a susceptibility gradient to histolysis was demonstrated for various regions of tail-skin. In this connection it was clearly shown that integument derived from anterior regions of the tail undergoes a more rapid rate of histolysis than is true of integument derived from more posterior regions. It was, consequently, thought of interest to determine whether or not any differences existed between the rates of regeneration of tail-skin derived from various anterior-posterior levels of the tail. Work on amphibian tail regeneration by several investigators seems to have given rise to rather diverse results and interpretations. Morgan (1906), working with the salamander *Diemyctylus viridescens*, and Ellis (1908, 1909), on the anuran *Rana clamitans*, seem to agree in a general way that the rate of regeneration of new portions of the tail is directly proportional to the distance the tail is cut off from the tip. In other words, the more distal the cut, the slower the regeneration. Conversely, Comes (1928), working with the anuran *Discoglossus pictus*, states that regeneration of the tail fin diminishes anteriorly and with the age of the animal. Speidel (1929), however, using several types of anurans and salamanders, reports no differences in the rate of resorptive or regenerative processes of the tail fin at anterior, middle, or posterior levels following thyroid treatment. Since the investigations of Morgan and Ellis on tail regeneration concern regeneration of all tissues of the tail, and the work of Comes and Speidel concerns the rate of tail fin regeneration *in situ,* it seemed of interest to the writer to inquire into the possible regenerative rate differences of only one type of tail tissue as might be demonstrated by transplantation to foreign regions. By so doing, it was thought possible to demonstrate whether a difference in regenerative rate was due to the association of the tissue with a definite level of the tail and adjacent tissues or whether the particular tissue in question possesses a specific rate of regeneration typical of integument of that region. As far as the writer is aware, the present paper constitutes the first attempt to explain the above points, especially as regards the rate of regeneration of histolyzing tail-skin taken from various levels of the tail.

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[1] This paper, together with Parts I and II of this series of studies, was submitted to the Graduate School of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, April 1, 1932.

The general methods of attack which were followed in the present investigation were briefly as follows: The integument from four levels (anterior-posterior) of the tail of normal larvæ was transplanted, autoplastically, to the back. Following this procedure, artificial metamorphosis was induced and the engrafted integument removed at certain stages of histolysis and transplanted, homeoplastically, to the back or tail of normal individuals. Subsequent macroscopic and microscopic observations were made of the various transplants at certain definite intervals as regeneration progressed. The following series of transplantations, which will be described in detail in the respective sections, were performed:

Series 1. Autoplastic skin transplantations from four regions of the tail to the back. (One hundred and twenty cases of four transplants each.)

Series 2. Skin transplants (previously transplanted in Series 1 and in various stages of histolysis) transplanted homeoplastically to the tail or back. (Two hundred and twenty-five cases of two transplants each.)

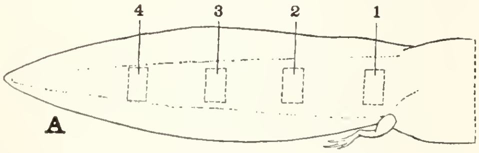
Series 3. Homeoplastic skin transplantations of four regions of the tail of normal larvæ to the tail or back of normal larvæ. (Thirty-two cases, control series.)

This investigation was undertaken at the suggestion of Dr. O. M. Helff, to whom the writer wishes to express his appreciation for his helpful suggestions, kindly advice, and criticism during the course of the work.

MATERIAL AND METHODS

The stock used for all operations was *Rana pipiens* larvæ obtained from ponds in the vicinity of Cold Spring Harbor, Long Island, during the months of June and July, 1931. The animals were taken to the laboratory and placed in a large tank supplied with fresh running water. It was found advisable to let the stock remain in the large tank from four to five days under laboratory conditions before selecting individuals to be used for experimental purposes. By doing this, only those individuals which survived the new environmental conditions would be selected. These animals ranged from 70 to 90 mm. in body length with hind limbs between 3 and 12 mm. in length. All experimental individuals were normal larvæ in all respects and remained as such until induction of artificial metamorphosis following the autoplastic operations. All operated animals were kept in individual aquaria and maintained under constant laboratory conditions.

With the exception of a small number, it was found necessary to anæsthetize all animals used for transplantation purposes. The animals were anæsthetized in a 0.05 per cent aqueous solution of chloretone for five to ten minutes, depending on individual differences and the temperature of the solution. These anæsthetized animals remained inactive sufficiently long to carry out operative procedures and insure adhesion of the transplants and their cut edges.



In the first series of experiments, autoplastic transplantations were made consisting of the removal of tail-skin from regions 1 and 3 or from regions 2 and 4 (text figure *A*). These portions of integument were then carefully shaped into rectangles measuring approximately four millimeters in width and seven millimeters in length. Two similar-sized and -shaped rectangular portions of integument from the medial region of the back were next removed and the tail-skin transplants from regions 1 and 3 or 2 and 4 were then transplanted to the denuded areas of the back. As regards the orientation of the grafts, the linear sequence of transplants number 1 and 3 or 2 and 4 were alternated in 50 per cent of the cases. In other words, graft number 1 or 2 was grafted to the more anterior wound area, leaving graft number 3 or 4 to be transplanted to the remaining posterior wound area; while in other cases graft number 3 or 4 was placed more anteriorly. After the above transplantations were made, the animal was placed in a Petri dish containing a small amount of water. This procedure allowed the grafted areas to be exposed to the air and hastened the adherence of the cut edges. Following this, the animals were placed in individual aquaria containing water which was kept at a temperature ranging from 18° to 20° C. After five days, the time allowed for sufficient healing of the graft to take place, precocious metamorphosis was induced by the feeding of desiccated thyroid.

During larval transformation, daily macroscopical observations were made of the above individual transplants to note the reduction in area of the grafts. When the grafted integument had been reduced in area approximately eighteen per cent, a number of grafts originally derived from tail regions 1, 2, 3, and 4 were removed and transplanted, homeoplastically, to normal larvæ. The same procedure was carried out for a like number of grafts when the reduction in area had reached approximately 50 and 80 per cent. In making the homeoplastic transplantations, the histolyzing graft was removed and placed on previously denuded areas of the back or lateral regions of the tail of normal larvæ. Following these transplantations, the normal larvæ were again placed in individual aquaria and kept as such under constant laboratory conditions. At intervals of 8, 11, 14, 17, 20, 23, and 26 days, following the homeoplastic operations, representative individuals possessing the transplanted integument of each of the four tail regions which had previously been reduced in area by 18, 50, or 80 per cent, were removed from their individual aquaria and preserved *in toto* in Bouin's Picro-Formol fluid for subsequent sectioning and histological study of the grafts.

A second series of homeoplastic transplantations was also made. The technique consisted of removing rectangular portions of tail-skin from regions 1, 2, 3, and 4 of normal larvæ. These normal skin transplants were then placed on denuded areas of the back or tail of other normal larvæ. Individuals of this series were also preserved for histological study of the transplants at 8, 11, 14, 17, 20, 23, and 26-day intervals following transplantation. This series was designed as a control to make certain that histolysis does not occur in larval skin following homeoplastic transplantation to normal larvæ.

RESULTS

The Process of Degeneration and Regeneration in Tail Integument

1. *The normal integument.*—The normal tail integument in the particular species studied (*Rana pipiens*) presents a macroscopical picture differing in appearance from integument of body regions. It appears

much more translucent and is also decidedly more delicate in texture, being injured and torn more readily than integument of the side or back. It is also attached more rigidly to the underlying musculature as compared with back-skin. The pigmented areas are regularly distributed over the entire tail region and appear to be less intense in depth of shade as compared with similar areas of the back.

As to histological structure, tail-skin is composed of an epidermis, a corium, and a layer of subcutaneous connective tissue. The epidermis is composed of several layers of cells, the outermost of which consists of a single layer of small, flattened cells forming the cuticle. The more basal cells of the epidermis are for the most part cuboidal in shape with round or oval-shaped nuclei. The epidermal cells basal to the cuticle are usually arranged into two or three irregular layers. The corium, which is separable into two definite layers in most regions of the body, differs markedly in this respect in tail integument. The outer, comparatively loose layer (stratum spongiosum), so typical of side and back integument, is almost obliterated in tail integument. This layer is represented, however, by a thin layer of pigment cells lying directly beneath the epidermis. The lower layer of the corium (stratum compactum) is composed of dense connective tissue, the fibers running in a wave-like course parallel to the surface. The characteristic much convoluted appearance, typical of this layer in body regions, is absent in tail integument. This layer is, moreover, much thinner as compared with the condition found in back integument. These corionic modifications, typical of tail-skin, are responsible for the latter's relative thinness and more delicate structure as compared with integument of body regions. The subcutaneous connective tissue forms a loose layer underneath the stratum compactum. This layer is quite vascular, containing large numbers of blood cells, chiefly lymphocytes. It is also considerably thinner than the subcutaneous connective tissue layer beneath back integument.

2. *The degenerative process.*—The same histolytic characteristics are exhibited for all four anterior-posterior transplants of tail integument when the degree of resorption, as measured by reduction in surface area, is the same. Therefore, in the explanations to follow, the process of degeneration will be described only as observed in tail-skin from region number 1 (text figure *A*). When the transplant had been reduced in surface area 18 per cent, no apparent macroscopical differences as compared to normal integument of the tail could be noted. The pigmentation remained normal throughout this period of resorption (Fig. 3). However, when the transplants had been reduced 50 and 80 per cent, respectively, a decided change in depth of shade could be noted.

In other words, the greater the degree of histological disintegration, the darker in color the transplant became (Figs. 1 and 2). This increase in depth of coloration proceeded from the margins of the transplant toward the center as histolysis progressed.

In examining histological sections of the tail-skin transplants at various stages of area reduction, the first signs of histolysis are concerned with the structure of the stratum compactum. When the transplant had been reduced in surface area 18 per cent, the stratum compactum had lost its wavy appearance; the fibers becoming more or less dissociated so that a very disorganized picture is presented (Fig. 5). With this dissociation, lymphocytes make their appearance, apparently migrating from the subcutaneous tissue into the compactum layer. These lymphocytes are particularly abundant at the edges of the transplant, where fusion has occurred with the surrounding integument of the back. At this stage of transformation the epidermis appears to be thickening, especially near the central region of the graft.

When the engrafted integument had undergone resorptive changes amounting to 50 per cent of its original surface area, the stratum compactum was entirely obliterated leaving only scattered pigment cells, lymphocytes, subcutaneous connective tissue, and the epidermal layers. An interesting histological feature of the graft in this stage of histolysis is the increased thickness of the epidermal portion, which appears to be several layers thicker at this time (Fig. 6). The basal layer of this

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PLATE I

Explanation of Figures

Figs. 1–5. *BI*, adjacent back integument; *P*, pigment masses; *TST*, tail-skin transplant; *E*, epidermis; *SS*, stratum spongiosum; *SC*, stratum compactum; *CT*, subcutaneous connective tissue; *L*, lymphocytes; *TT*, boundaries of transplant; *BV*, blood vessel.

Fig. 1. Macroscopic appearance of tail-skin graft histolyzed 50 per cent its original surface area.

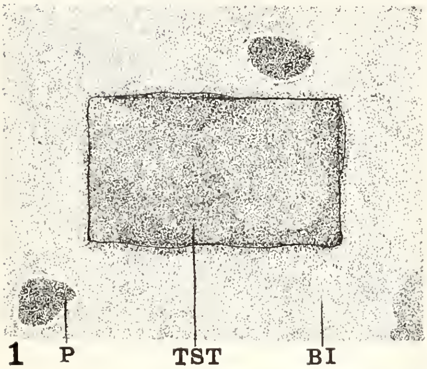
Fig. 2. Macroscopic appearance of tail-skin transplant histolyzed 80 per cent its original surface area.

Fig. 3. Macroscopical picture of normal tail-skin transplant as it appears five days following autoplastic transplantation.

Fig. 4. Histological section through autoplastic tail-skin transplant and adjacent back-skin at a time when the graft had histolyzed 80 per cent (surface area reduction). The stratum compactum is obliterated. The epidermal layer is beginning to dissociate and lymphocytes are migrating into this particular region. There is also a slight condensation of pigment cells basal to the epidermal portion of the transplant.

Fig. 5. Histological section through autoplastic tail-skin graft and adjacent back integument when the transplant had histolyzed 18 per cent. The stratum compactum has lost its wavy appearance and is becoming more or less dissociated. Subsequent lymphocytic invasion is shown following this dissociation. The epidermal portion of the graft appears normal at this time.

PLATE I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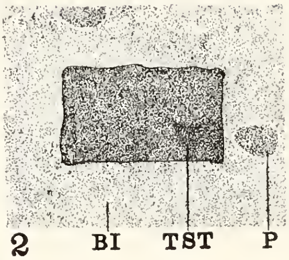
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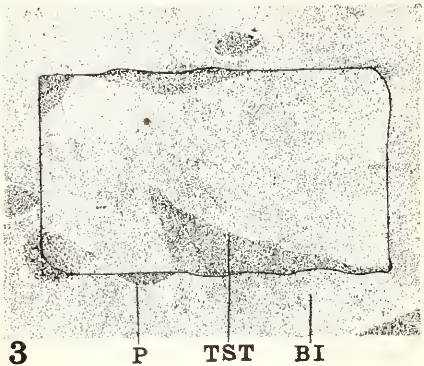
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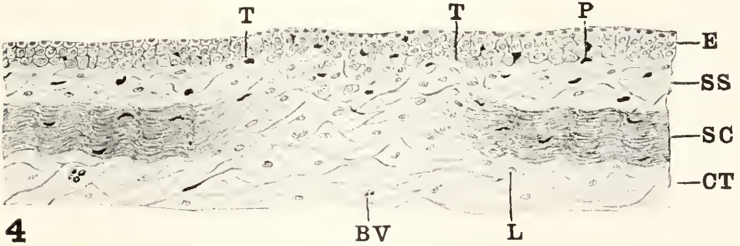
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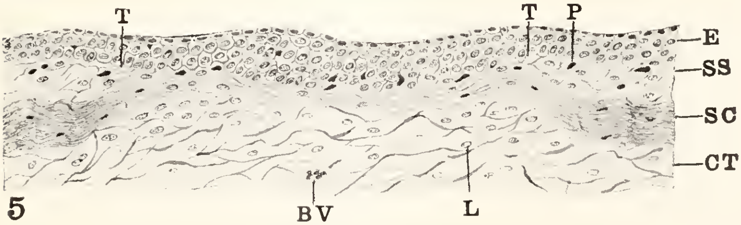
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thickened epidermis contains cells which have hypertrophied and assumed a more columnar shape. In sections of tail-skin grafts, where the original surface area had been reduced approximately 80 per cent (Fig. 4), the previously thickened epidermis, as illustrated in Figure 6, is now very much thinner. Sections show a separation and basal migration of groups of these epidermal cells. With this epidermal dissimilation there is a gradual invasion inward of all layers of the surrounding back integument. This invasion probably places the graft under a certain degree of peripheral pressure. The lymphocytic cells are now found scattered throughout all the various layers of the graft, including the epidermis. The cuticle is still intact at this stage and apparently is the last layer to exhibit a histolytic change.

The above histological and macroscopical findings show that the integumentary grafts exhibit histolytic processes which are identical with those typical of histolyzing tail integument, *in situ,* during normal metamorphic tail atrophy. In this connection, it may be stated that a more complete study of the histological processes which take place during histolysis was not attempted; only those sections taken at definite periods, when surface area had been reduced 18, 50, and 80 per cent, having been studied. It may be stated here, however, that the microscopical pictures of tail-skin histolysis as thus observed compare favorably with those of Helff (1926) and of Lindeman (1929*a*).

PLATE II

Explanation of Figures

FIGS. 6–10. Histological sections through back integument including homeoplastic tail-skin transplants originally derived from the most anterior regions of the tail. The sketches illustrate successive steps in the histological regeneration of the transplants which had previously undergone partial histolysis following autoplastic transplantation. *E,* epidermis; *SS,* stratum spongiosum; *SC,* stratum compactum; *CT,* subcutaneous connective tissue; *P,* pigment masses; *TT,* boundaries of the transplant; *L,* lymphocytes; *BV,* blood vessel.

FIG. 6. Section through autoplastic tail-skin graft and adjacent back integument showing condition of transplant when histolyzed 50 per cent (surface area reduction). The stratum compactum is nearly obliterated. The epidermis is increased in thickness with its more basal cells somewhat hypertrophied.

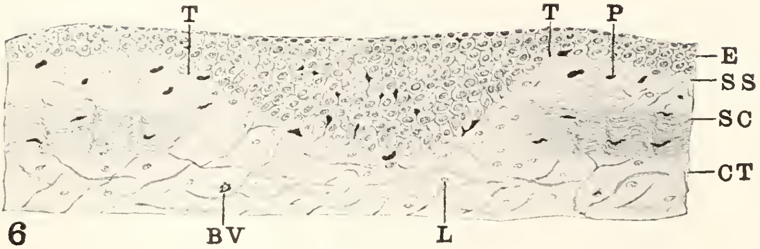
FIG. 7. Eight days following homeoplastic transplantation. The first signs of regeneration as shown by a condensation of the epidermis.

FIG. 8. Eleven days following homeoplastic transplantation. Basal cells of the epidermis breaking off and separating from the main portion of that layer.

FIG. 9. Fourteen days following homeoplastic transplantation. Epidermis still dissociating in the basal layers. Evidence of a stratum compactum layer, its fibers being more or less loosely associated.

FIG. 10. Seventeen days following homeoplastic transplantation. Histologically, the transplant appears as normal tail integument.

PLATE II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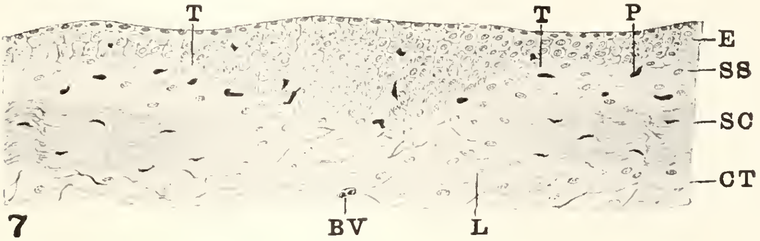
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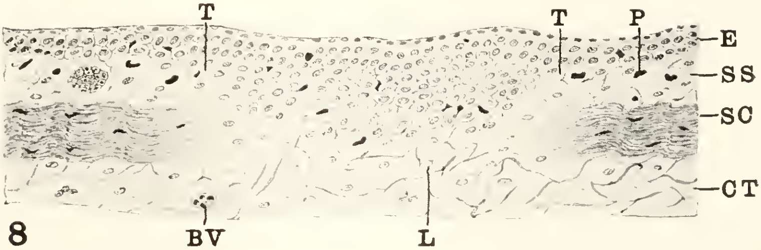
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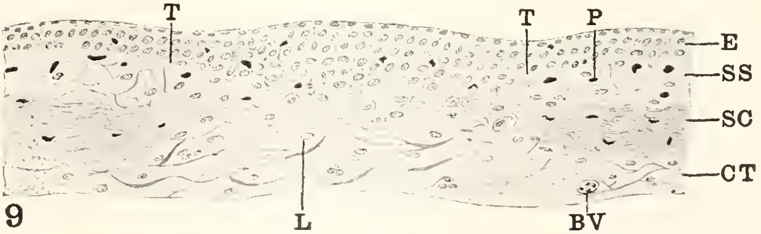
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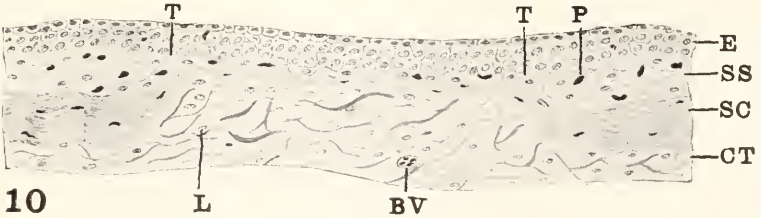
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3. *The regenerative process.*—Tail-skin transplants reduced in surface area 50 per cent were used as typical forms in the following description of the regeneration of histolyzing tail-skin. The histological pictures (Figs. 6–10) illustrate the process of regeneration from the time of transplantation (homeoplastic) of the histolyzing graft to normal larvæ, to the time when the graft in question had again assumed a normal histological picture. Sections showing the intermediate periods were taken at three-day intervals following the five-day period allowed for healing of the homeoplastic transplants.

In macroscopical appearance (Fig. 1) the graft, prior to the homeoplastic transplantation, appears much darker in color than the normal integument of the tail region from which it was originally derived. This darkened coloration is especially noted as being more intense near the margins of the graft. The depth of coloration remained as such for approximately eight days following homeoplastic transplantation. From this time on the transplant began to assume a lighter color; the margins of the graft, however, always appearing darker as compared with the more central regions. Normal coloration of the integumentary transplant, however, was not attained until approximately fourteen days following the homeoplastic transplantation.

Several transplants from tail region 1 were sectioned at the time homeoplastic transplantations were usually made. Sections were also made of transplants after 8, 11, 14, 17, 20, 23, and 26 days following homeoplastic transplantation to normal larvæ. Histological examinations show that subsequent changes in the grafts are concerned with the building up of previously partially disintegrated structures to the condition typical of normal tail integument (Figs. 6–10). During this process the more internal or basal cells of the epidermis are either reduced in size or replaced by smaller cells during the early stages of regeneration. This process is later followed by a gradual dissimilation or breaking down of the basal layers of the epidermis, so that at approximately seventeen days following homeoplastic transplantation the resulting epidermis appears normal again. (See Fig. 10.) The stratum compactum shows no evidence of returning to normal until about the eleventh to fourteenth day. On the fourteenth day (Fig. 9) the fibers appear to be more or less condensed and are grouped into bundles. These loosely associated fibrous bundles contain a number of scattered epidermal cells in and around them. Subsequent observations made three days later (Fig. 10) show that the stratum compactum has assumed a wavy appearance and appears as a typical normal layer. Although the stratum compactum is only about one-third as thick as the corresponding layer in the surrounding back integument, the two unite and form a perfect union. Usually a decrease in number of lymphocytes in the immediate region of the regenerating tissue can be noted. Finally, the integument at the end of 17 days appears to be normal as regards all histological features. However, while emphasizing the return to normal of the transplant as regards histological structure, it must be stated that the surface area remains the same throughout the entire process of regeneration. Thus the completely regenerated transplant in question is only approximately 50 per cent as large in actual surface area as the original autoplastic graft.

A comparison was also made of the process of regeneration in integument derived from tail region 1, as described above (50 per cent reduction), to similar transplants in which 18 per cent and 80 per cent reduction had occurred. In the transplants which had reduced but 18 per cent in area, the typical normal histological structure was observed in from 11 to 14 days, whereas in the case of grafts reduced 80 per cent in area, approximately twenty days were necessary for normal reconstitution to take place. This indicates, clearly, that the greater the degree of histolysis as based on surface area reduction, the longer the time necessary for complete histological regeneration.

Comparative Regenerative Rate of Integument from Four Tail Regions

In determining whether or not a susceptibility gradient to regeneration existed, it was necessary to make initial daily observations on the progress of histolysis of specific regional transplants, as well as subsequent histological examinations concerning the progress of regeneration. In these preliminary observations on tail-skin degeneration, the author was able to confirm previous work (Clausen, 1930) concerning an anterior-posterior gradient of susceptibility to histolysis. The earlier results were confirmed in that a regressive change in histolytic rate of tail integument from the most anterior region to the most posterior region of the tail was found to occur.

Daily macroscopical observations of the transplants from the four anterior-posterior regions of the tail revealed a decided difference in coloration during regeneration. It was noted that when graft number 1 had resumed its characteristic normal pigmentation at the end of 14 days following homeoplastic transplantation, graft number 4 remained apparently unchanged as regards coloration. Upon sectioning the transplants from regions 1 and 4, as well as those from the intermediate regions 2 and 3, a decided regional difference in the time taken for the grafts to complete the process of histological regeneration was noted (Table I). Table I presents the essential data obtained by the sectioning and making of a histological study of tail-skin transplants following their second or homeoplastic transplantation. However, it should be noted that this table illustrates only differences in regeneration rate based on complete histological reconstitution of the transplants. Other histological examinations made at successive three-day intervals following homeoplastic transplantation show approximately the same variation in regenerative rate between transplants originally derived from the four tail regions. In other words, there is found to be a difference in regenerative rate between transplants 1, 2, 3, and 4 for the attainment of any of the various stages of regeneration. This gradient in regenerative rate was found to hold true regardless of whether the partially histolyzed transplants were placed on the back or tail regions of the host

TABLE I

Regeneration of Tail-skin Transplants [2]

Regional Source *	Reduction in Area †	Time taken for return of normal histolytic picture			Average Time
		Case (1)	Case (2)	Case (3)	
	per cent	*days*	*days*	*days*	*days*
1	18	11	14	11	12
	50	14	17	17	16
	80	20	20	20	20
2	18	14	14	11	13
	50	17	17	20	18
	80	23	20	20	21
3	18	14	17	14	15
	50	20	23	20	21
	80	23	23	26	24
4	18	17	17	17	17
	50	23	23	20	22
	80	26	26	23	25

* Anterior-posterior level of tail from which transplant was originally obtained.

† Stage of histolysis (as determined by area reduction) at which second (homeoplastic) transplantation was made.

[2] To obtain sections of three transplants each from the four tail regions in three stages of histolysis which ultimately showed complete histological regeneration, it was necessary to section approximately one hundred and eighty transplants.

animals. In other words, the particular transplantation site of any one transplant was of no consequence to the rate of regeneration occurring. The difference in regenerative rate for complete reconstitution between transplants 1 and 4 was approximately five days; transplants 2 and 3, as compared with transplant 1, being intermediate in this respect.

Similar observations on a group of individuals used as controls, where normal integument of the four tail regions was transplanted homeoplastically to the tail or back of normal larvæ, were also made. The results obtained from this series of experiments serve to indicate, clearly, that the transplantation procedure itself does not influence the histolysis or regeneration of the integument, either as regards the histological processes involved or the rate of regeneration which occurs.

Discussion

Histological studies made from sections of histolyzing tail integument as described in the present paper tend to show that different integumentary layers do not histolyze at the same rate. In other words, if the causal histolytic influence is the same throughout all regions of the transplant, it is evident that various layers of the integument differ as regards their susceptibility to histolysis. It may also be stated here that evidences of greater resorption of peripheral regions of the transplant as compared with more central areas is probably due to the fact that lymphocytes appear to be more numerous in the former regions and hence the histolyzed cellular elements are more quickly removed at such points.

In considering the coloration of transplants at various stages of histolysis, it may be concluded that changes in coloration are probably due to analogous changes in distribution of the underlying pigment cells. In this respect it may be stated that the number of pigment cells per any given area is always greater in histolyzing integument as compared with normal skin of the same region. It is also quite probable that variable changes in thickness of the epidermal portion of the graft during histolysis may be concerned with external changes in coloration. Since a considerable portion of the pigment material lies basal to the epidermal layer, any change in thickness of the latter results in a displacement of the former in respect to its distance from the cuticular surface of the integument. Consequently, the thinner the epidermal portion, as illustrated by degree of histolysis (Fig. 4), the nearer to the surface the pigment cells become arranged; and hence the greater the intensity of integumentary coloration, viewed externally. Conversely, in the 50 per cent reduction stage (Fig. 6) a less intense coloration is typical of

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the more central portions of the graft. This condition is no doubt due to the fact that the greatly thickened epidermis forces the pigment layer an abnormal distance down and away from the surface of the integument.

The results of the present work indicate that there is an inherent difference in regenerative rate between integuments of different levels of the tail. Moreover, these results serve to emphasize that the rate of regeneration progressively increases from the posterior to the more anterior regions of the tail. Histologically, tail integument from various anterior-posterior tail regions appears to exhibit similar structural characteristics. On this basis, any morphological explanation for differences in regenerative rate can hardly be justified. It seems logical to assume, therefore, that the basis for such differences in regenerative rate must be a physiological one.

It is of interest to inquire at this time what factors operate to inhibit further histolysis of the homeoplastic transplants and thus permit regeneration to proceed. The results of the present and of previous work clearly indicate that histolysis is initiated by factors present in the blood stream only during larval metamorphosis. Assuming that regenerative tendencies are inherently present in the integument, we must then conclude that the histolytic effects are so powerful that any tendency towards regeneration is soon overcome and histolysis normally proceeds until the integument in question is completely destroyed. Upon transplantation, however, to a normal non-metamorphosing larva in which no histolytic blood factors are present, the integumentary transplant ceases to histolyze further. Concurrently, the inherent regenerative tendencies are no longer suppressed and the various histological elements are therefore capable of being regenerated.

The results of the present investigation seem to compare favorably with the earlier work of Morgan (1906) and Ellis (1908) in that the rate of regeneration increases from the distal to the more proximal regions of the tail. The results of Speidel (1929), however, indicate no change in regenerative rate of tail fin at various anterior-posterior levels. However, since it has been shown in the present paper that the regenerative rate differences between transplanted integument from various tail levels are not great and also since the work of Speidel concerned the regeneration of tail fin tissue *in situ,* it can readily be understood how the relatively small rate differences may have been masked by other physiological factors typical of the various tissues of the tail. The results of investigations by Comes (1928), which also possibly differ from the results as cited in this paper, may also be explained by the fact that his observations were concerned with regeneration of normal tail fin tissue *in situ.* Furthermore, it may be well to state here that Speidel and Comes were both primarily concerned with linear growth of the tissue or tissues in question. Therefore, since the results as presented in the present paper are concerned chiefly with regeneration of partially histolyzed tissue and deal mainly with histological regeneration, a direct comparison with that of the earlier work can hardly be made.

The present work finally suggests the possibility that the underlying musculature of various tail regions may also exhibit differences in regenerative rate following partial histolysis. It is also of interest to inquire as to the possible physiological factors associated with such differences in regenerative rate. The difference in regenerative potentialities of integument at various levels is either an inherent quality present during early embryonic formation or one acquired during some stage of early larval growth. Work designed to adequately explain the above points is being carried on in this laboratory at the present time.

Summary and Conclusions

1. Uniform-sized tail-skin grafts were secured from extreme anterior and posterior regions and from two intermediate areas and transplanted, autoplastically, to the backs of *Rana pipiens* larvæ. Artificial metamorphosis was induced and the engrafted integument removed at certain stages of histolysis and transplanted, homeoplastically, to the back or tail of normal larvæ. Following homeoplastic transplantations, anterior grafts invariably regenerated, histologically, with greater rapidity as compared with posterior integumentary grafts. Intermediate tail-skin grafts regenerated at proportionate rates.

2. Control homeoplastic transplantations of skin from four regions of the tail, when grafted to the tail or back of normal larvæ, clearly indicated that histolysis does not occur in larval tail-skin following transplantation to normal individuals.

3. Histologically, the process of degeneration illustrates a definite, orderly sequence. Dissociation of various layers begins prior to the appearance of lymphocytes in the histolyzing area. Lymphocytes probably function chiefly as phagocytes.

4. The regenerative process is first evidenced, histologically, in the epidermal layers of the graft. The stratum compactum is shown to be the last layer of integument to completely regenerate.

5. It is concluded that an anterior-posterior gradient in regenerative rate is true for tail integument following partial histolysis. Apparently, differences in regenerative rate of integument at different levels of the

tail are inherent qualities. Possible physiological factors associated with such differences, however, are still undetermined.

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THE POLARITY OF THE EGG OF URECHIS CAUPO

ALBERT TYLER

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, and the William G. Kerckhoff Marine Laboratory, Corona del Mar, California)

It has been previously noted (Tyler, 1931) that the indentation of the unfertilized egg of *Urechis* marks the pole. This conclusion was based on observations of a small number of eggs in which the relation of the point of extrusion of the polar bodies to the indentation was followed. More recently Taylor (1931) reported some observations, made by Miss Vesta Holt on a slightly larger number of eggs, the results of which show no relation between the indentation and the point of extrusion of the polar bodies. This question has therefore been re-investigated on a larger scale.

The method of making such observations and the precautions to be taken have been previously described (Morgan and Tyler, 1930; Tyler, 1931). It consists of mounting a drop of eggs in sea water on a slide within a square of vaseline. The vaseline serves to support the cover-slip at a much greater distance above the slide than the diameter of the egg. It also serves to seal the preparation and thus prevent evaporation of the sea water from the drop. The drop makes contact with both the slide and the cover-slip but can be adjusted so as not to touch the vaseline. This last precaution, however, was probably unnecessary since the vaseline used was found to be non-toxic to the eggs. The eggs were fertilized in a dish and a sample transferred after one minute or less to the vaseline slide. They stay in position quite well on the vaseline slide, and the extra spermatozoa on the surface serve as markers by means of which any rotation of the egg can be detected. The eggs develop on the vaseline-slide into perfectly normal swimming larvæ of two or more days of age.

The indentation of the unfertilized *Urechis* egg is a concavity similar to that which would be produced by pushing in the surface of a rubber ball at one point to about forty-five per cent of the original diameter. The indentation disappears after fertilization, and, as has been elsewhere described (Tyler, 1932), the *Urechis* egg exhibits three types of behavior in regard to its rounding out. The egg may round out initially in three minutes after fertilization, become indented again at six minutes, and round out finally at ten minutes; or the indentation may not entirely disappear the first time; or the indentation may disappear at three minutes and not reappear again. The eggs followed in the observations reported here were mainly of the first and third types. Eggs from freshly collected animals appear to be generally of the last type.

A total of 590 eggs were followed in the observations reported here. The results again show that the indentation marks the pole of the egg for the type that rounds out at three minutes and does not dent in again. For the type in which the indentation reappears this second indentation sometimes does not come back in the same place, but the point of extrusion of the polar bodies is almost invariably determined by the position of the second indentation.

Often eggs are obtained that have two indentations. The point of extrusion of the polar bodies usually coincides with one of the indentations. Such eggs generally lie with one of the indentations down so that they might be mistaken for eggs with only one indentation. This complication entered into some of the observations, as is evident from the results given in Table I.

TABLE I

Relation between Indentation and Point of Extrusion of Polar Bodies

Series A = isolated eggs with only one indentation.
Series B = random sample of eggs.

Divergence	Series A	Series B
0°–10°	92	72
10°–60°	12	20
60°–180°	10	35

In Table I are given the results of observations on freshly removed eggs from freshly collected animals. The eggs of Series A were all isolated and examined individually before being used in order to make sure that they had only one indentation. The results show 81 per cent of almost exact coincidence between the innermost central point of the indentation and the point of extrusion of the polar bodies; 11 per cent were close (10° to 60° divergence); and 8 per cent were 60° to 180° off. For the eggs of Series B no special precautions were taken to insure their possessing only one indentation, although they were obtained from batches of eggs most of which showed only one indentation. Out of 127 eggs in this series 35 were 60° to 180° off. But of these 35 eggs the position of the polar bodies was found to be below the egg. This result when compared with that of Series A may then be taken to mean that where coincidence was not obtained the unfertilized egg possessed another indentation lying below the egg and thus not visible. There is also the possibility as indicated below that in these cases the indentation reappeared in a different position, namely, on the underside of the egg, but this is not likely since their sister eggs did not exhibit the reappearance of the indentation.

In Table II are given the results obtained on the type of egg in which a second indentation appears at six minutes after fertilization and then disappears at ten minutes. The point of extrusion of the polar bodies diverges by 60° to 180° from the central point of the first indentation in 41 per cent of the cases (117 eggs). But in 98 of these eggs the position of the second indentation was also noted and its central point was found to coincide with the point of extrusion of the polar bodies in 82 of the cases. Of the 130 cases of coincidence between the first indentation and the point of polar body extrusion, the position of the second indentation was noted in 87 cases and found to be identical with the first in 84 of them. In addition there were 66 eggs in which only the position of the second indentation was noted. The last column in Table II gives the results of all the observations in which the relation of the second indentation to the pole was noted. Here there are only 12 per cent of cases in which the divergence is very large.

TABLE II

Relation between indentation and point of extrusion of polar bodies. Eggs in which a second indentation appears.

Divergence	First Indentation	Second Indentation
0°–10°	130	210
10°–60°	36	12
60°–180°	117	29

It appears then that for this type of egg the position of the second indentation is a much better index of the pole of the egg than the first. This is particularly obvious in those cases in which the position of both indentations was observed—where the position of the polar bodies coincided with that of the first indentation it almost invariably coincided with the second, and where it did not coincide with the first indentation it coincided with the second in the great majority of the cases. However, even in the case of the first indentation there are 46 per cent of cases of coincidence (0° to 10° divergence), whereas a random distribution of the polar bodies with respect to this indentation would probably not give better than 8 per cent coincidence.

The main question involved in this work is whether or not the polarity of the *Urechis* egg is determined before fertilization, as appears to be the case for most animal eggs. For the type first discussed the results clearly show that polarity is already established in the unfertilized egg, and the pole is marked by the position of the indentation. For the type in which a second indentation appears after fertilization there is no reason to doubt that polarity is established before fertilization although the position of the pole is not as clearly marked by the first indentation as by the one appearing after fertilization.

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THE

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STUDIES OF THE MITOTIC FIGURE

I. Chaetopterus: Central Body Structure at Metaphase, First Cleavage, After Picro-Acetic Fixation

HENRY J. FRY

(From Washington Square College, New York University, and the Marine Biological Laboratory, Woods Hole)

I. Purpose of the Study

Several years ago, in studies of central bodies in *Echinarachnius* eggs, the writer reached the conclusion that in the cytasters, spermasters, and first-cleavage figures of this egg the central body is a coagulation product of the area of focalized rays and spindle fibers, having no existence as an individualized body in the living cell (Fry, 1928, etc.).

To ascertain whether this situation exists in other forms, a number of studies have now been made of various cell types, including spermatocytes, oöcytes, recently fertilized eggs, early and late blastomeres and somatic cells, in various organisms, ranging from ceolenterates to vertebrates. The central bodies in these cells exhibit such a wide diversity in behavior that the writer has made a provisional classification of centrioles and centriole-like bodies, which is discussed later (p. 181). Each class is identified by some phase of behavior not shared by the others. In the case of each type of cell investigated the purpose was to find out to which class the centriole belongs. After all of these results have been reported, the interrelations of the different classes will be discussed in a later paper.

The material of the present study, which is the first of the group just mentioned, is the egg of *Chaetopterus pergamentaceus.* Mead (1898) described typical centrioles in this egg which maintain genetic continuity from one cell cycle to the next. Wilson (1930) recently re-examined the original preparations of that work and confirmed Mead's observations. Some years ago the writer attempted to duplicate Mead's findings, using Boveri's picro-acetic reagent. Mead states that of the fixatives he employed this gave the best results. In the majority of the cells the writer studied, however, the astral centers were disrupted. A group of experiments was therefore carried out, using this same reagent, for the purpose of ascertaining what phase of technique was responsible for Mead's demonstration of centrioles on the one hand, and the writer's inability to repeat the work on the other. Study was confined to metaphase asters of first-cleavage figures, because in general central bodies are most readily demonstrated about the time of metaphase, even if they are not present at earlier or later phases; and the mitotic figure of first cleavage has the advantage of its unusually large size.[1]

II. Methods

Treatment of Living Eggs

Prior to fertilization, the eggs were divided into three lots,[2] and fertilized at five-minute intervals, e.g., 9:55, 10:00, and 10:05. They were later mixed and the average time, e.g., 10:00, was regarded as the time of fertilization. Hence when eggs are fixed for the purpose of securing them at metaphase, which occurs in about 52 minutes at 21° C., there is a " spread " of stages from early prophase to late anaphase, and many eggs are in metaphase even if development has been retarded or accelerated in any one egg-set.

After fertilization the eggs were placed in a 1000 cc. crystallizing dish filled with sea water and kept covered to avoid evaporation and consequent modification of osmotic pressure. The water was changed every ten minutes and the eggs were gently stirred every two or three minutes. This avoids possible effects of overcrowding, keeps the oxygen supply normal, and removes metabolic wastes. The dish was immersed in a water bath and kept at a temperature of 21° ± .5. About five minutes before fixation the eggs were transferred to small Stender dishes. Just prior to fixation the water was poured off from each dish, leaving at the bottom a dense mass of eggs, together with a minimum amount of sea water, which necessarily accompanies the eggs. All egg-sets used showed more than 95 per cent first cleavage.

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[1] Appreciation is expressed to Miss Sara J. Reynolds for her aid as research assistant in the work.

[2] The parapodia were cut up in a small amount of sea water, then placed on wet cheesecloth drawn taut over an empty bowl, and water was gently squirted through the mass until all eggs were released. Thereafter the water was changed every five minutes for the first half hour, during which maturation reaches metaphase of the first division. The eggs of two or more females were used in each egg-set, and they were fertilized about half an hour after they were secured. The sperm suspension was prepared by adding one drop of thick seminal fluid to about 10 cc. of sea water. Several drops of this were added to the eggs in 250 cc. of water. The exact dilution of the sperm suspension is unimportant, since polyspermy rarely occurs.

Fixation and Slide-making

The picro-acetic reagent employed in the experiments was made according to Boveri's formula (1887, p. 11): 99 parts of a saturated solution of picric acid which has been diluted with two volumes of water, and one part of glacial acetic acid. In experiments where this formula was modified the details are given in each case. Unless otherwise stated, eggs were run up in the usual manner, sectioned at a thickness of 5 μ, and stained in Heidenhain's hæmatoxylin.

Making Observations

It is well known that the two asters of the first-cleavage figure in *Chaetopterus* eggs differ in size (Fig. 1). In this paper only the larger aster of each figure was studied, in order to exclude possible variations due to differences in astral size.

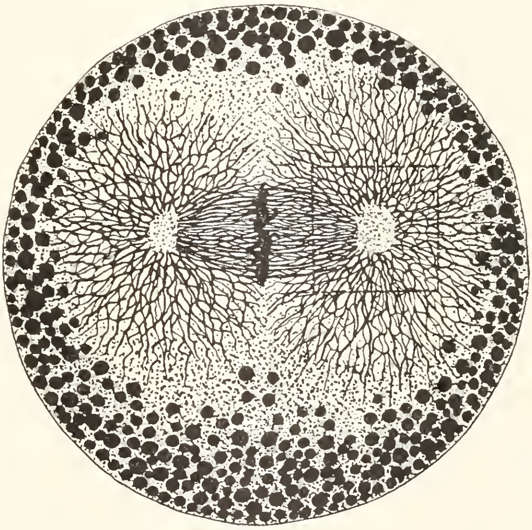

FIG. 1. The metaphase first-cleavage figure in *Chaetopterus* eggs after using Boveri's picro-actetic fixation.

The size difference between the two asters is apparent. The area outlined by the dotted lines is that part of the figure illustrated in the charts. There is here shown that type of structure occurring most frequently after using Boveri's picro-acetic fixation.

Metaphase is here regarded as the time when chromosomes are aligned in a flat plate. Eggs in late prophase, when the chromosomes are in a broad irregular group, and in early anaphase, when they are just beginning to separate, were excluded.

Only the mid-section of the large aster was studied. The sections at the left and right were always examined in order to be sure which was the mid-section. If any section was missing that egg was discarded.

A 4 mm. high dry objective was used in making a list of readings. When several types of central bodies occur on the same slide, as is usually the case, one may stand out more distinctly than the others, especially when such an objective is used. To make sure that a random sample of the egg population of each slide was secured, and to eliminate unconscious selection of any class, the slide was searched systematically by the use of a mechanical stage, and every metaphase figure was listed until the desired number was obtained. These cells were then studied under critical lighting conditions at magnifications of 600 or 900 ×, using an objective having a numerical aperture of 1.4 and a similarly corrected condensor.

Illustrations

In this study, as well as in those to be reported later, it is as important to illustrate the details of ray structure as of central bodies, since the major conclusion of the work is that the structure of the one is closely related to that of the other. Four types of illustration were tried: photographs made with white light, photographs made with ultra-violet light, wash drawings, and ink drawings.

Photographs made with white light show adequately the structure of the central body, but, owing to the nature of the photographic process,[3] they fail completely to show the finer details of ray structure so obvious to the eye.

Through the cooperation of Dr. F. F. Lucas, of the Bell Telephone Laboratories, ultra-violet photographs were made of the asters of *Chaetopterus* and several other species. They will be reported in a later paper, since they will have more significance if discussed after the regular cytological studies have been completed. Such photographs show delicate detail with maximum clarity; but until the technique has been simplified and made less expensive, they cannot have wide-spread use in cytological studies. Hence they are not employed in this group of papers as the regular mode of illustration.

[3] If, for example, a photograph is made of a series of alternating dark grey and light grey lines which have a relative intensity of 10 to 1, the relative intensity of the images of those lines on the negative is 2 to 1. This phenomenon has no practical consequence when photographing such objects as chromosomes or typical centrioles which are in distinct contrast to the surrounding material. But wherever there are only slight differences in intensity, and where the pattern is a delicate one, as in the case of ray and inter-ray materials, a photograph is capable of showing only a vague and unsatisfactory image of the actual structure.

Drawings of any kind, whether wash or ink, have the disadvantage of possibly showing unconscious over-emphasis of certain points or inadvertent omission of others; but they can show delicate details not reproduceable by white light photography, and they have the distinct advantage of showing what the eye sees at various levels of the preparation. In most cytological papers drawings of both astral rays and spindle fibers are frankly schematized, since the exact and literal delineation of the complicated ray pattern presents practically insurmountable obstacles—obstacles not associated with illustrations of most cell components. In many cases, e.g., Mead's wash drawings of *Chaetopterus* asters (1898), the metaphase rays are shown as straight, whereas they are actually more or less undulating. If ink drawings are used, as is often done, where rays are usually shown as black lines and inter-ray materials are not shown at all, the illustration has a degree of contrast much greater than that in the preparation. Furthermore, completely homogeneous areas, such as some large centrosomes, can be shown only by closely-placed dots, which give a granular effect unlike that of the original structure.

Wash drawings are unquestionably more pleasing aesthetically than ink drawings, for the use of several tones of grey and black results in an illustration having a general appearance more like that of the original preparation than is possible when only black is used. But both types can be either accurate or inaccurate in delineation of structure; and when such complex detail as the exact configuration of astral rays is illustrated both wash and ink drawings are inevitably more or less schematized. To show differences in the coarseness and the shape of rays in various astral types, ink drawings are capable of giving as adequate a picture of structural variations as are those made with the wash technique.

Ink drawings have been selected as the mode of illustration in this group of papers, because they can be embodied in charts, reproduceable with zinc plates, which are impracticable when wash drawings are used. Thus variations in astral structure can be related in a graph-like manner to the modifications of various experimental conditions, and there is apparent at a glance the relative frequency of each type under each condition.

Only the central region of the aster is shown in each drawing—an area indicated by the dotted lines in Fig. 1, page 151. If entire cells were shown at the magnification used, 1000 ×, they would occupy too much space to be included in charts.

The drawing of each of the twelve astral types occurring in this study was made from a specific cell, chosen because it was typical of the class. Each drawing, therefore, not only delineates a single " best " cell, but it also represents the type.

Terminology

Following Wilson's usage, (1928, pp. 30 and 672–675) the term *centriole* is used to indicate a minute darkly-stained granule-like body; the term *centrosome* refers to a larger, more variable structure that often surrounds the centriole or may exist by itself. A *centrosphere* is a large vacuolar area at the astral center. The term *central body* is a general one; it may apply to any or all of these structures, and hence includes all configurations from a minute granule to a large empty area.

Mead uses these terms with different meanings: what is here termed a *centriole* he calls a *centrosome*, and what is here termed a *centrosome* he calls a *centrosphere*.

III. EXPERIMENTS

GROUP A. TECHNIQUE STANDARD: BOVERI'S PICRO-ACETIC FIXATION AND REGULAR SLIDE-MAKING PROCEDURE

Experiment 1. The Relation Between Central Body Structure and Ray Structure

In this experiment the 243 eggs studied were selected at random from slides of various egg-sets which had been fixed, sectioned, and stained under supposedly similar optimum conditions. Preliminary examination having shown several types of central bodies present, an attempt was made to ascertain whether or not these variations in central body structure could be related to other structural modifications of the mitotic figure. To that end the large metaphase aster in each cell was analyzed with reference to the following points:

1. The central body
    - A. The centrosome
        1. Physical structure (empty, containing more or less scattered materials, or evenly homogeneous)
        2. Size and shape
        3. Degree of demarcation from ray area (demarked distinctly, doubtfully, or not at all)
        4. Stain in contrast to that of ray area (lighter, similar, or darker)
    - B. The centriole
        1. Number
        2. Location in the centrosome
        3. Size
        4. Shape (regular or irregular)
        5. Contour (smooth or rough)
        6. Stain in contrast to that of the centrosome

- C. Granules, other than centrioles, occurring near the astral center
    - 1. Number
    - 2. Size
    - 3. Location
    - 4. Similarity to cytoplasmic granules
- II. The rays [4]
    - A. Coarseness (very coarse, medium coarse, delicate, or vague)
    - B. Shape (rippled or serpentine, undulating or almost straight)
    - C. Occurrence of small vacuole-like areas among the rays
- III. The spindle
    - A. Size
    - B. Shape of tip (pointed, rounded, or intermediate)
    - C. Structure of fibers in contrast to that of astral rays

The various types of centers occurring after Boveri's picro-acetic fixation constitute an unbroken series: the central area may be completely empty; it may contain either small amounts of scattered material, sometimes arranged like the walls of vacuoles, or more abundant material, distributed regularly or irregularly; finally, it may be an evenly-filled, homogeneous region. These centers are about $5 \times 6\,\mu$ in size, although there is much variation here.[5] Whether such a series is illustrated by few or many drawings is purely arbitrary. In Chart I three classes are shown: "empty," "scattered," which contain more or less irregularly distributed material, and "even," or homogeneously filled centers, which merge gradually with the ray area and are stained like it.

The various ray configurations also constitute a continuous series: at one extreme they are "rippled" or serpentine; at the other they are "undulating" or almost straight; the intermediate type can best be described as "slightly rippled." The rippled rays have many minute clear spaces, like tiny vacuoles, among their deep curves; the undulating ones have few or none.

Thus when *Chaetopterus* eggs in metaphase are fixed with Boveri's reagent there is a definite relation between central body structure and ray structure: all asters with rippled rays have disrupted centers that are either empty or scattered; all those with undulating rays have even centers that are homogeneously filled. There are no exceptions to this relationship. Asters with slightly rippled rays, which are intermediate between these two classes, show all types of centers.

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[4] It is difficult to select concise terms that describe clearly the differences in the coarseness of rays and in their shape. For example, the use of "very coarse" in contrast to "medium coarse" is somewhat clumsy, but it is necessary to express two degrees of coarseness as contrasted with the condition described by the term "delicate." The terms "rippled" and "undulating" were selected because the former suggests the idea of short sharp curves, in contrast to the long gentle curves suggested by the latter.

[5] Even among cells in metaphase which are alike in the general structure of rays and central bodies there is considerable variation in the size of the center It is practically impossible to secure accurate measurements in most cases, because the central body is demarked from the ray area either vaguely or not at all. In each type, therefore, the cell selected for illustration has a central body of about the average size for its class, based on attempted measurements of about twenty figures of that type. There is also much variation in the shape of the centers: some are round, others quite elongate, others intermediate. The cells selected for illustration as representative for each type have central bodies that are intermediate in shape, being but slightly elongate.

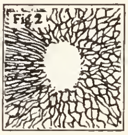
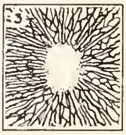
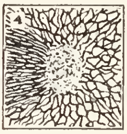
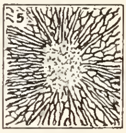
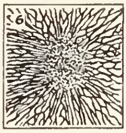
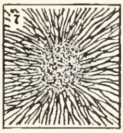
CLASSES OF CENTRAL BODIES	CLASSES OF RAYS		
	Rippled:	Slightly Rippled	Undulating
Empty	 62 Asters	 14 Asters	
Scattered	 66 Asters	 34 Asters	
Even		 30 Asters	 37 Asters

CHART 1. THE RELATION BETWEEN RAY STRUCTURE AND CENTRAL BODY STRUCTURE UNDER OPTIMUM CONDITIONS

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation, and the standard slide-making procedure. *Result:* Central body structure is related to ray structure: centers are "disrupted", *i.e.*, empty or containing scattered material, if rays are rippled in shape; centers are "even", *i.e.*, homogeneously filled and stained like the ray area, if rays are undulating. Centrioles are not demonstrated.

The slightly rippled rays are obviously intermediate between rippled and undulating rays. Since typically rippled rays are always associated with disrupted centers (empty or scattered) and typically undulating ones with evenly filled centers, the members of this intermediate, or slightly rippled group will hereafter be included with either the rippled or the undulating class, depending upon which group the aster most resembles. Thus the four classes of asters illustrated in Chart 1, in which the centers are empty or scattered and rays either rippled or slightly rippled (Figs. 2–5) will hereafter be regarded as but minor variations of a single major type, and represented in later charts by an illustration showing a disrupted (scattered) center and rippled rays (Fig. 4). Similarly, the two classes with even centers and rays either slightly rippled or undulating (Figs. 6 and 7) will hereafter be regarded as variations of another major type, represented in later charts by an illustration showing an even center and undulating rays (Fig. 7). These two major types of asters are but two of twelve that occur in this investigation, all of them illustrated in Chart 7 (p. 177). The type with disrupted centers is designated as *1B* and that with even centers as *2B*. The basis of classification is discussed on page 176.

Very rarely, *i.e.*, in 16 of the 243 asters studied, one or more granules, like the smaller ones present in the cytoplasm, occur in the even type of center. But since they vary in size, location, and staining capacity, they could not be interpreted as centrioles. These random granules are not the structures Mead illustrated; the kind of central body he described was not produced in this experiment, in which Boveri's picro-acetic reagent was used in the usual way.

Experiment 2. The Effects of Uncontrolled Factors in the Handling of Different Egg-sets Under Optimum Conditions

The purpose of this experiment was to learn whether the percentages of disrupted and even centers (Types *1B* and *2B*) occurring in one set of eggs handled under optimum conditions are the same as those occurring in other egg-sets run under supposedly similar conditions but on different days. Table I shows counts of these two types of centers in five different sets, each sample including from 33 to 67 eggs. These data are arranged in the order of increasing percentages of the disrupted type.

When Sets 1 and 2, 2 and 3, 3 and 4, and 4 and 5 are compared, there are only minor differences in the percentages of the two central body types present, and these could be explained by the relatively large errors always involved in reporting small samples. But when Sets 1 and 3, 2 and 4, and 3 and 5 are compared, the differences are large enough to suggest some cause beyond error of sampling. And when Sets 1 and 4, 1 and 5, and 2 and 5 are contrasted it is obvious that the discrepancies are too great to be explained by errors of sampling alone; they must be due either to differences between the living eggs of the various sets prior to fixation, or to uncontrolled modifications of technique. When these relations are analyzed statistically, by determining the value of P according to Pierson's method (1924, p. lxx) the existence of factors which cause differences, other than errors due to small samples, is convincingly demonstrated.[6] The approximate values of P for this material are shown in Table I.

Three problems present themselves: (1) to secure more data concerning the relation between central body structure and ray structure; (2) to explain the cause of the difference in the relative numbers of disrupted and even centers present in different sets after picro-acetic fixation under optimum conditions; and (3) to ascertain what variation of the picro-acetic technique Mead used to demonstrate typical centrioles. The experiments which follow were planned in an attempt to secure information on these points.

Group B. Technique Modified: Slide-Making Procedure Varied; Boveri's Picro-Acetic Fixation

Experiment 3. The Effects of Varying the Depth of Stain (Heidenhain's Hæmatoxylin)

It is conceivable that a center containing considerable irregularly scattered material might, if lightly stained, be listed as belonging to the disrupted (scattered) type, while the same center, if darkly stained, might appear to be evenly filled and would then be counted as an even type. In that event, the differences in the relative numbers of disrupted and even centers in the five sets of the previous experiment might be due to differences in depth of stain.

In that experiment the eggs were stained a deep blue color with Heidenhain's hæmatoxylin. Table II repeats the data of that experiment at the left, and also shows, at the right, the numbers of disrupted and even centers which occurred when eggs of the same five sets were stained a very pale blue. In each set all experimental conditions were identical, in so far as they could be controlled, except the depth of stain.

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[6] Pierson's method determines whether a given sample of objects (the 67 eggs of Set 1) having a given number of one class (40 disrupted centers) and a given number of a second class (27 even centers) does or does not belong to the same population as another sample (the 55 eggs of Set 2) with different numbers of the same classes (36 disrupted centers and 19 even centers). These relations are expressed in terms of P. Thus the value of P for Sets 1 and 2, as well as for Sets 2 and 3, is 0.4, meaning that there are 4 chances out of 10 that each pair belongs to the same population, and indicating that the differences between them are not significant. At the other extreme, however, the value of P for Sets 1 and 4 is 0.02, and for Sets 1 and 5 it is 0.001, showing that the chances are, respectively, one out of 50 and one out of 1000, that each pair belongs to the same population. The differences between these sets are therefore significant.

TABLE I

The effects of uncontrolled factors in the handling of different egg-sets under optimum conditions. Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation, and the standard slide-making procedure. *Result:* Some factor or factors are involved which modify significantly the numbers of the two central body types in the different egg-sets.

Egg-Set Symbol	Number of Cells Studied	Distribution of Astral Types in Each Set		P
		1 B (Disrupted)	2 B (Even)	
1	67	40 60%	27 40%	
				0.4
2	55	36 65%	19 35%	0.1
				0.4
3	48	34 71%	14 29%	0.001
				0.1
4	33	26 79%	7 21%	0.01
				0.1
5	39	35 90%	4 10%	

It is apparent, both by inspection and by the statistical determination of the value of *P*, that the depth of stain with Heidenhain's hæmatoxylin does not modify significantly the percentages of the two central body types occurring in any one egg-set after Boveri's picro-acetic fixation. This is especially obvious when comparing the totals.[7]

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[7] This fact does not indicate that various depths of stain would be equally ineffective in modifying the appearance of other central body types, to be described later.

TABLE II

The effects of varying the depth of stain (Heidenhain's hæmatoxylin). Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation. The slide-making procedure was standard except that egg-sets reported at the left in the table were stained darkly and those at the right were stained lightly. *Result:* Differences in depth of stain do not significantly modify the numbers of the two central body types occurring in the different egg-sets.

EGG-SET SYMBOL	EGGS STAINED DARKLY			P	EGGS STAINED LIGHTLY		
	Number of Cells Studied	Distribution of Astral Types in Each Set			Number of Cells Studied	Distribution of Astral Types in Each Set	
		1 B (Disrupted)	2 B (Even)			1 B (Disrupted)	2 B (Even)
1	67	40 60%	27 40%	←0.8→	36	21 58%	15 42%
2	55	36 65%	19 35%	←0.9→	42	27 64%	15 36%
3	48	34 71%	14 29%	←0.6→	44	33 75%	11 25%
4	33	26 79%	7 21%	←0.7→	48	39 81%	9 19%
5	39	35 90%	4 10%	←0.1→	73	61 84%	12 16%
TOTALS	242	171 71%	71 29%		243	181 74%	62 26%

Experiment 4. The Effects of Other Modifications of the Slide-making Procedure

Wide variations as to the speed with which the eggs are passed through the alcohols and xylol, the temperature at which they are embedded, and the thickness at which the ribbon is sectioned, do not change the appearance of the central bodies when Boveri's picro-acetic reagent and Heidenhain's hæmatoxylin are used.

The effects of modifying one other phase of the slide-making procedure were also studied—accidental overheating of the ribbon when it is warmed to bring about its expansion. Different samples of ribbon were variously treated during this operation: in one the ribbon was expanded in the usual manner, remaining opaque without melting throughout the operation; another was so overheated that the ribbon melted and became transparent for a moment; in a third the same situation was produced but maintained for a longer time; finally, one slide was so extremely overheated that the ribbon not only melted but bubbled and gave off vapors. In the latter case a number of the eggs have a peculiar glassy appearance in the region of the spindle, the inner zone of the astral rays, and the central body area (Fig. 8). This type, produced by faulty technique, is readily recognized and could not be confused with the types occurring under usual conditions.

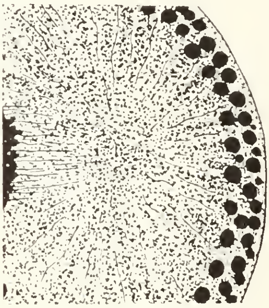

FIG. 8. The glassy type of astral center caused by overheating the ribbon when expanding it.

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation. The slide-making procedure was standard, except that the ribbon was overheated when expanding it. *Result:* The central body area, the inner part of the ray region, and the spindle have a glassy appearance.

It can therefore be concluded that when *Chaetopterus* eggs are fixed with Boveri's reagent, the inevitable slight variations in the slide-making procedure do not modify central body structure. While this conclusion applies only to the disrupted and even types, it is probable that it also holds true for the other types to be described.

GROUP C. TECHNIQUE MODIFIED: PICRO-ACETIC FIXATION VARIED; REGULAR SLIDE-MAKING PROCEDURE

Experiment 5. The Effects of Varying Simultaneously the Amounts of Picric and Acetic Acids

When eggs are added to the fixative in a vial, even the densest egg suspension includes some sea water, which slightly dilutes the reagent. The present experiment deals with the effects of a series of dilutions, beginning with one part of distilled water and 99 parts of reagent, and ending with 99 parts of water and one part of reagent. To test-tubes containing 25 cc. of each dilution were added 0.5 cc. of eggs. The amount of sea water included with the eggs was so small, compared with the 25 cc. of diluted fixative, that the effects of any further dilution could safely be ignored. The eggs fixed at 99 per cent, 90 per cent, 75 per cent, 50 per cent, 10 per cent and 1 per cent strength were from one egg-set; those fixed at 30 per cent, 20 per cent, 15 per cent, and 5 per cent were from another egg-set.

Chart 2 shows the percentages of the central body types occurring after fixation with each dilution. When the reagent is from 99 per cent to 50 per cent strength the disrupted and even types previously described (1*B* and 2*B*) are the only ones present, their numbers varying at the different dilutions. At 30 per cent strength two new types appear, one having a vaguely delimited centrosome stained darker than the ray area, but without a centriole (4*A*), and one with a similar centrosome but containing one or two centrioles (5*A*). Between 30 per cent and 5 per cent strength these are the major classes. At 5 per cent strength a new type of aster makes its appearance, similar to the one with an even center so frequently described except that it has very delicate rays (2*C*). At 1 per cent strength the asters are very small and vague and their centers are undifferentiated from the ray area. Cytasters are also present,[8] and the chromosomes are abnormal.

The relation between ray structure and central body structure is again obvious, and additional evidence on this point is supplied by the new types. (1) If rays are fixed vaguely, the central area of the aster is entirely undifferentiated from the peripheral part (2*D*). (2) If rays are distinct and rippled the center is disrupted (1*B*). (3) If rays are distinct but undulating, the astral center is filled, its structure varying with the coarseness of the rays, as follows: When rays are either delicate or medium coarse, the center is a homogeneous centrosome, about $5 \times 6\,\mu$ in size, not delimited from the ray area, and stained like it (2*B* and 2*C*). When rays are very coarse there are two classes of centers: (*a*) a centrosome, about $4 \times 5\,\mu$ in size, vaguely delimited from the ray area, more darkly stained, and containing no centriole (4*A*); and (*b*) a similar centrosome but with a centriole, either single or double (5*A*). The possible significance of these facts will be discussed after the data of all the experiments have been presented.

The writer reported these results of diluting the reagent to Pro-

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[8] As is well known, cytasters are usually produced by methods which bring about artificial parthenogenesis in unfertilized eggs. Occasionally, however, they have been described in normally fertilized eggs. It is possible that some of these were produced by the use of a dilute reagent, as an accidental result of the mode of fixation.

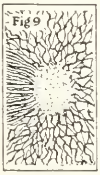
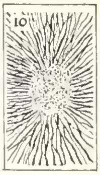
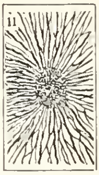
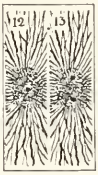
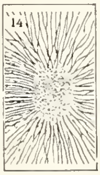
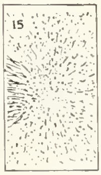
EXPERIMENTAL DATA			DISTRIBUTION OF ASTRAL TYPES AT EACH DILUTION					
DILUTION FORMULÆ		NUMBER OF CELLS STUDIED	*Type* 1B	2B	4A	5A	2C	2D
Boveri's Picro-acetic Reagent (c.c.)	Distilled Water (c.c.)		Fig 9 	10 	11 	12 13 	14 	15 
99	1	34	32 94%	2 6%				
90	10	34	29 85%	5 15%				
75	25	46	31 67%	15 33%				
50	50	82	80 98%	2 2%				
30	70	39	5 13%	5 13%	12 31%	17 43%		
20	80	52	6 .2%	6 12%	19 36%	21 40%		
15	85	41	1 2%	4 10%	10 25%	25 61%	1 2%	
10	90	47			6 13%	41 87%		
5	95	47			19 40%	17 36%	11 24%	
1	99	40						40 100%

CHART 2. THE EFFECTS OF VARYING SIMULTANEOUSLY THE AMOUNTS OF PICRIC AND ACETIC ACIDS

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with Boveri's reagent diluted to various degrees with distilled water. The slide-making procedure was standard. *Results:* (1) Central body structure is related to ray structure, depending upon the coarseness and shape of the latter. (2) Mead (1898) demonstrated centrioles by diluting the reagent. (3) Uncontrolled dilution effects, resulting from the manner in which eggs are added to vials under usual conditions (Exp. 2) are probably responsible for the variations in numbers of the central body types in different egg-sets.

fessor Mead, suggesting that possibly a comparable dilution might have been responsible for the centriole phenomena he found, and hence for the writer's failure to repeat the work with a full-strength reagent. Professor Mead replied that dilution did occur in his experiments, that the results in different preparations were not uniform, and that in many eggs the centers were disrupted. His failure to mention this dilution in his paper is undoubtedly due to the fact that he attached no significance to it—a situation which occurs almost universally in the case of many supposedly minor variations in different steps of the cytological technique. It is worth noting, however, that diluting the reagent is the only modification of the many employed in this investigation which demonstrates centrioles.

Where Mead used sea water to dilute the fixative, distilled water was used in the present work. It is probable that both kinds of dilutions produce similar results, for centrioles can be demonstrated after dilution with either. It is possible, however, that certain differences may exist.

Varied dilution of the reagent explains the occurrence of disrupted and even types of central bodies side by side on the same slide, as well as the variations in their relative numbers in various egg-sets supposedly fixed and run up in the optimum manner (Table I, p. 159). Uncontrolled dilution effects, differing from vial to vial and modifying the central area in asters, are brought about by slight differences in the manner in which eggs happen to be added to the reagent in the vial. If eggs are squirted quickly from the pipette into the vial containing the reagent the mixing is practically instantaneous, but the eggs are handled rather violently. Furthermore, the dish containing the living eggs may become contaminated if the tip of the pipette, which must be held close to the vial when the eggs are added, is splashed with minute droplets of the reagent. For these reasons, eggs are usually allowed to drop gently out of the pipette. Under such conditions the eggs and any sea water accompanying them are more or less segregated near the top of the vial for the first few seconds, the reagent mixing with them from below. It will be shown later (Experiment 9) that fixation occurs within one second; hence the eggs added to the vial are exposed during the first second to various dilutions of the fixative, depending upon whether they happen to be at the top or at the bottom of the mixture of eggs and sea water just added to the reagent.

The data of Chart 2 show that with few exceptions fixation with a full-strength reagent produces disrupted centers (1*B*), and that progressively greater dilutions of the reagent result in filled centers (2*B*, 4*A*, and 5*A*). In the preceding experiment (Table I) such effects were inadvertently produced by the manner in which the eggs of the five egg-sets were added to their vials. In Set 1 a considerable percentage of the eggs undoubtedly received a somewhat dilute fixation, since 40 per cent of them have even centers; but those of Set 5 were added in such a manner that only 10 per cent of them were so fixed.

The effect of varied dilutions also explains why, under usual conditions of fixation, about 70 per cent of the centers are disrupted, the remaining ones being even (Table II). Since *Chaetopterus* eggs frequently stick to the bottom of the vial, the writer has heretofore used a narrow vial (9 mm. wide, inside diameter, and 4.5 cm. high), because in it the eggs pile up on top of each other, and few of them touch the bottom. Prior to fixation these vials contained from 4 to 4.5 cc. of reagent; at the time of fixation from 0.5 to 1.0 cc. of eggs were added. The strength of the reagent thus varied from 80 per cent to 90 per cent, the average being about 85 per cent. In the five sets fixed under these usual conditions, 71 per cent of the eggs have disrupted centers and 29 per cent even ones—a result which is comparable to that of the set fixed at 75 per cent strength in the dilution experiment (Chart 2), where the percentages are 67 and 33.

In this dilution experiment the major differences in types and numbers of central bodies occurring at any given strength of the reagent are without doubt primarily the result of the varied dilutions. Within each set, however, the numbers and proportions of central body types were probably also modified by the exact way in which the eggs were added to the reagent. Until the results of this experiment were known the writer had not realized what radical effects can be produced by dilution. Had the eggs been expelled suddenly from the pipette into the fixative instead of being dropped in gently, not only in the present experiment but in the others as well, it is probable that in many cases the numbers of types would have been reduced and their relative percentages somewhat altered.[9]

The fact that dilution of Boveri's picro-acetic reagent causes such marked differences in the structure of rays and central bodies does not mean that this would be true of all cells fixed with any dilute reagent. However, it is probable that the cells of various species are susceptible to dilution effects in various fixatives. For example, although *Echinarachnius* eggs ordinarily show large pleuricorpuscular central bodies when fixed with various full strength reagents, including Bouin's, this fluid diluted with 90 parts water produces a minute centriole surrounded by a homogeneous centrosome. The second paper on *Chaetopterus* eggs will demonstrate that the effects of diluting certain chemically diverse reagents are similar to those of picro-acetic dilutions, although the details differ in each case.

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[9] The existence of such an uncontrolled cause of variation in central body types makes it useless to study large egg samples in each group for the sake of reducing the error inherent in small samples. For this reason the number of eggs studied at each variation is seldom more than 50, the average being 36. But although uncontrolled dilution effects may modify the percentages of any type occurring in the eggs of any vial, they do not alter the relation between central body structure and ray structure, which always holds true, without exception.

Whether dilution effects have played an unsuspected rôle in central body studies of other species remains to be ascertained by future investigations. It may be that in many eggs extensive dilution of the reagent does not modify astral structure. One egg, however, that may be affected in this way is that of *Ascaris* (Fogg, 1931 and many earlier papers); its chorion is so impermeable that it can live in certain strong reagents for hours. When the one usually used, a chloroform-alcohol-acetic mixture, does finally penetrate, it is quite possible that only a very dilute amount of the reagent as a whole, or of one of its components, actually brings about the fixation. This can be determined only if a technique can be developed whereby *Ascaris* eggs can be subjected to instantaneous fixation with full-strength reagents.

Another egg where dilution effects probably occur is that of *Drosophila* (Wilson and Huettner, 1931). It too is impermeable to the reagent. Therefore it is pricked at one end to permit the fixative to enter, and thirty to sixty seconds are required for it to traverse the egg. This may explain the variations found in different mitotic figures in the same stage. In different eggs the variations may be determined by the size of the puncture, which governs the rate at which the reagent enters; but similar effects also occur in different parts of the same egg, probably depending on various dilution effects at different distances from the point of puncture. Here again it would be interesting to compare the results produced by a technique permitting instantaneous, full strength fixation, with those of the present method.

The fact that a certain configuration is produced by a given dilution of a reagent but is not shown when that fixative is used full strength, argues neither for nor against the validity of that coagulation product. Such differences, produced by different dilutions of the same fixative, may have neither more nor less significance than differences produced by chemically diverse reagents. Nevertheless, if certain configurations are produced only by a diluted reagent, that fact must be kept in mind.

The manner in which significant variations are produced in *Chaetopterus* eggs by diluting the reagent suggests that certain precautions should be followed generally in fixing eggs. The amount of reagent should be large enough to prevent any appreciable dilution by the addition of any fluid accompanying the eggs. Furthermore, the eggs should be added in such a way as to effect instantaneous mixing, in order to prevent inadvertent dilution effects during the first second, when the eggs are fixed.

Experiment 6. The Effects of Varying in Turn the Amounts of Picric and Acetic Acids

In the preceding experiment the amounts of both acids in the reagent were progressively and simultaneously reduced. The present experiment was carried out in order to study the effect on central body structure when each acid in turn is kept constant while the other is varied.

Four egg-sets, run on different days, were used in this experiment. Each illustration in Chart 3 shows the astral configuration resulting from fixation with a different combination of picric and acetic acids. The figures, grouped in four series, are arranged in the order of percentage by weight of both acids present in each case. In series A, shown by the top horizontal row of figures, the picric acid, used as a saturated solution, was kept constant at about 1.2 per cent, and the acetic varied from 0.007 per cent to 20 per cent, the picric here being three times as strong as in Boveri's picro-acetic formula. In Series B, shown by the second horizontal row of figures, the saturated picric acid solution was diluted with two volumes of water (a mixture designated on the chart as " ⅓ picric "). This is the same concentration used in Boveri's reagent, which is 0.4 per cent by weight. In Series C, illustrated by the vertical line of figures near the center of the chart, the acetic acid was kept constant at 1 per cent, its concentration in Boveri's formula, while the picric was varied from 0.01 per cent to 1.2 per cent.[10] In Series D, shown by the oblique row of figures in the lower left-hand corner of the chart, both acids were varied, beginning with picric at 0.4 per cent when acetic is at 1.0 per cent, and ending with picric at 0.003 per cent and acetic at 0.01 per cent. This last series, which was reported in the previous experiment, Chart 2, is included here for purposes of comparison with the other three series. Chart 3 also shows the central body type present after fixation with a 1 per cent solution of acetic acid containing no picric acid (Fig. 43), as well as that occurring after fixation with " ⅓ picric," containing no acetic acid (Fig. 44).

Table III shows the various formulæ employed. In calculating the

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[10] In Chart 3, the picric acid of Series A and B is spoken of as being constant, but this is only relatively true. In Series A, for example, while the acetic acid was varied from 0.007 per cent to 20 per cent, the picric was varied only from 1.2 per cent to 1.1 per cent and thus was relatively constant. The same situation applies to Series B. In Series C the acetic acid actually was kept constant, since 1 cc. of it was added to 99 cc. of different dilutions of picric acid solution.

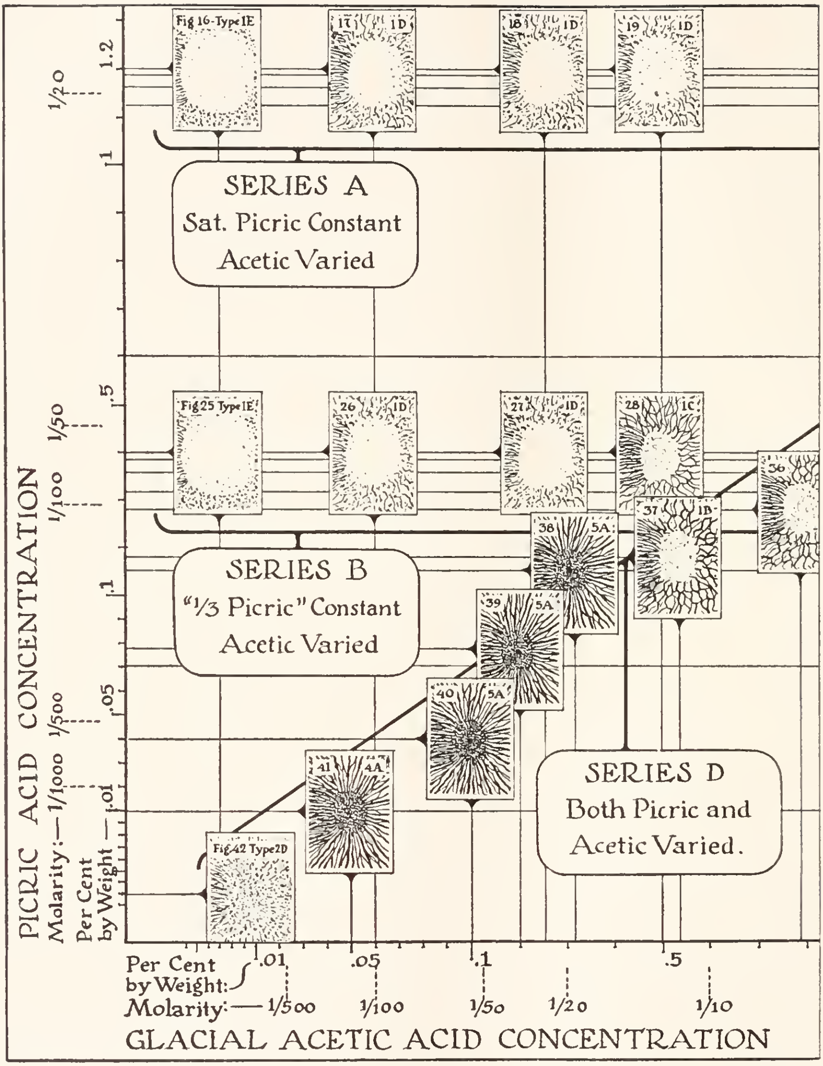
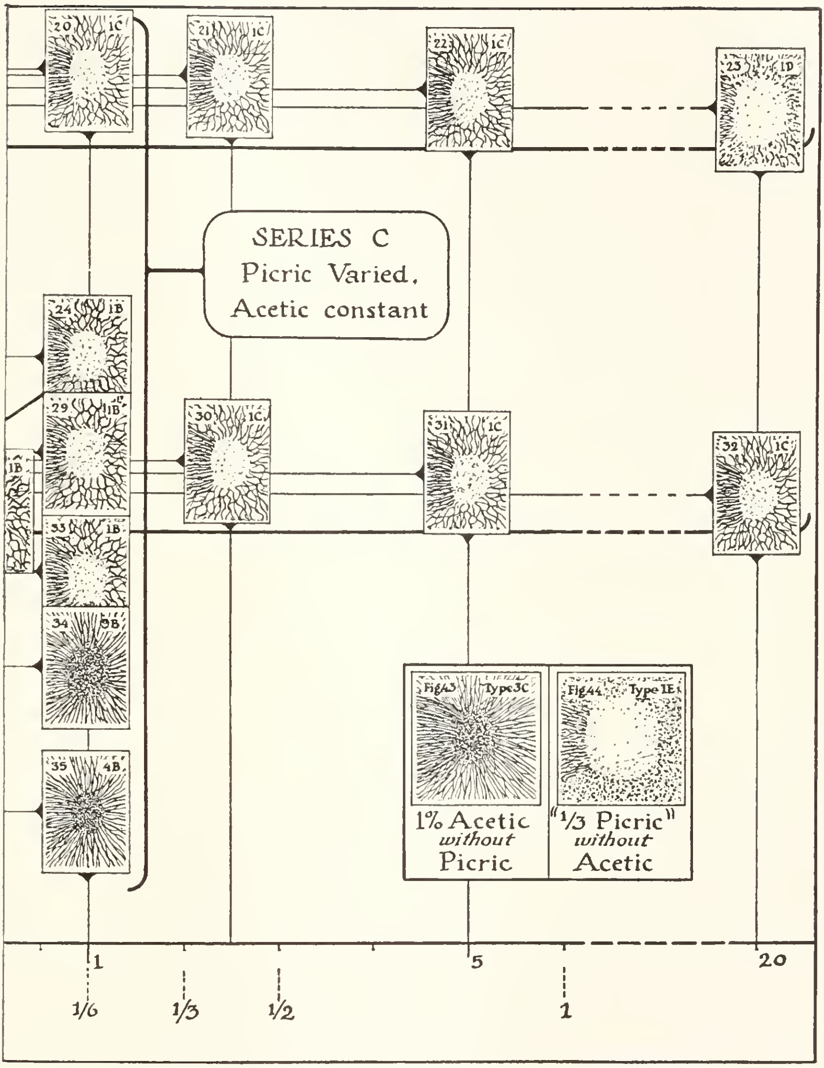


CHART 3. THE EFFECTS OF VARYING IN TURN

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with various modifications of the picro-acetic reagent. In some the amount of picric acid was kept constant while the acetic acid was varied (Sets A and B); in others the reverse was done (Set C). The data of the preceding


20 1C
21 1C
22 1C
23 1D
SERIES C
Picric Varied,
Acetic constant
24 1B
29 1B
1B
30 1C
31 1C
32 1C
33 1B
34 3B
35 4B
Fig43 Type3C
Fig44 Type 1E
1% Acetic
without
Picric
"1/3 Picric"
without
Acetic
1
5
20
1/6
1/3
1/2
1

THE AMOUNTS OF PICRIC AND ACETIC ACIDS

dilution experiment are included for comparison (Set D). The slide-making procedure was standard. *Result:* Only under a very narrow set of conditions are centrioles demonstrated and rays fixed in an undulating and very coarse configuration.

percentage by weight in each formula, 1 cc. of glacial acetic acid was regarded as weighing 1 gram, since its specific gravity is 1.05. The effect upon the solubility of the picric acid (1.22 grams are soluble in 100 cc. of water at 20° C.) caused by changes in room temperature, as well as the possible effect of the presence of acetic acid in the mixture, was ignored. Extreme accuracy in making up the formulæ is meaningless, since slight unknown dilutions were brought about in each case by the addition of small amounts of sea water with the eggs at the time of fixation. The molarities of the two reagents are also shown; they too are only approximately correct.

Table III

Formulæ of the picro-acetic reagents used in the experiments reported in Charts 2 and 3

Series A SATURATED PICRIC CONSTANT: ACETIC VARIED			Series B "1/3" PICRIC CONSTANT: ACETIC VARIED			Series C PICRIC VARIED 1% ACETIC CONSTANT					Series D BOTH PICRIC AND ACETIC VARIED		
Sat. Picric	Acetic	Fig.	Sat. Picric Diluted with 2 Parts Water	Acetic	Fig.	Formulæ of Picric Solutions		Picric Sols.	Acetic	Fig.	Picro-Acetic Reagent	Water	Fig.
						Pts. Sat. Picric	Pts. Water						
cc.	*cc.*		*cc.*	*cc.*				*cc.*	*cc.*		*cc.*	*cc.*	
99.99	.007	16	99.99	.007	25	100	0	99	1	20	99	1	29
99.94	.06	17	99.94	.06	26	50	50	99	1	24	75	25	36
99.75	.25	18	99.75	.25	27	33	67	99	1	29	50	50	37
99.5	.5	19	99.5	.5	28	12	88	99	1	33	30	70	38
99.	1.	20	99.	1.	29	6	94	99	1	34	20	80	39
97.5	2.5	21	97.5	2.5	30	1.5	98.5	99	1	35	10	90	40
95.	5.	22	95.	5.	31						5	95	41
80.	20.	23	80.	20.	32						1	99	42

In order to accommodate the drawings, the abscissa of the chart, which shows the percentage of acetic acid, is drawn to various scales in its different parts; *i.e.*, 1 cc. = ½ inch; 0.1 cc. = ¼ inch; 0.01 cc. = ⅛ inch; 0.001 cc. = 1/16 inch. The ordinate showing the percentages of picric acid has been similarly adjusted.

In Series A, B, and C, where there is only slight variation in structure at each modification of the reagent, the illustration on the chart represents the type which is most abundant, based on a study of about 20 eggs in each case. In Series D, where the variation is considerable, large numbers of eggs were studied, but again only the major class occurring after fixation with each dilution is shown, since all of the types occurring are illustrated in Chart 2.

Six new astral types occur in this experiment: (1) one having a disrupted center and delicate rippled rays (*1C*, Figs. 20–22, 28, and 30–32); (2) one with a dense center, stained more darkly than the ray area but not delimited from it, with medium coarse, undulating rays (*3B*, Fig. 34); (3) a similar aster with delicate rays (*3C*, Fig. 43); (4) one with a slightly demarked center, darker than the ray region, with medium coarse, undulating rays (*4B*, Fig. 35); (5) one with a disrupted center accompanied by vague or doubtful rays (*1D*, Figs. 17–19, 23, 26, and 27); and (6) a similar center accompanied by a ray area that is entirely non-radial (*1E*, Figs. 16, 25, and 44).

The results of this experiment are as follows: (1) A typical centriole and its accompanying very coarse undulating rays can be demonstrated only when the percentage by weight of the picric acid is between about 0.01 per cent and 0.2 per cent, and the acetic is at the same time between about 0.05 per cent and 0.4 per cent. Other types of central bodies and ray configurations also occur within this range. (2) If the concentration of the acetic acid is extended to about 2.0 per cent and the picric is held within the range just mentioned, the centers, which are accompanied by medium coarse, undulating rays, are darker than the ray area and are either undemarked from it, *i.e.*, dense (*3B*, Fig. 34), or slightly demarked from it (*4B*, Fig. 35). (3) If the concentration of the picric acid is extended beyond about 0.2 per cent, and that of the acetic beyond about 2.0 per cent, the centers are disrupted and the rays rippled, being either coarse (*1B*), delicate (*1C*), vague (1D), or absent (*1E*), depending upon the formula used. (4) If both picric and acetic acids are very dilute, the picric being less than about 0.005 per cent and the acetic less than about 0.01 per cent, the aster shows only a vague radial organization (*2D*, Fig. 42).

This experiment establishes the fact that the demonstration of a centriole, which is always accompanied by very coarse undulating rays (*5A*), occurs only when the picric and acetic acids are simultaneously within a certain narrow range of concentration.

Experiment 7. The Effects of Varying the Hydrogen Ion Concentration of Boveri's Reagent

Chart 4 shows the effects of the addition of varying amounts of normal sodium hydroxide solution to Boveri's picro-acetic fixative. The pH reported in each case[11] was modified to a slight but unknown degree by the addition of the small amount of sea water which always accompanies the eggs at the time of fixation. In this experiment, however, the egg samples were kept unusually small, so as to change the pH as little as possible. All eggs used in this experiment were from the same egg-set.

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[11] The hydrogen ion concentration was determined electrometrically by Mr. Delafield Dubois, who used an apparatus with glass electrodes.

Amounts of normal sodium hydroxide solution listed in Chart 4 were added to 40 cc. of Boveri's reagent.

The two common central body types previously described occur after using the reagent at its natural pH, 2.2. When the pH is increased to 3.9 the rays are largely suppressed, but the centers remain the same (*1D* and *2D*). When the pH is 4.7, 5.3, or 5.7, rays are undulating and delicate, while the centers are either even (*2C*) or dense (*3C*). In all sets except the one in which the pH was unmodified the mitotic figures are very small, the chromosomes poorly fixed, and the cytoplasm, except in the region of the mitotic figure, crowded with large, darkly-staining granules. A definite relation between ray structure and central body structure is again shown.

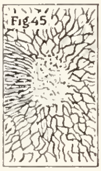
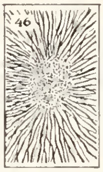
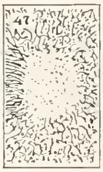
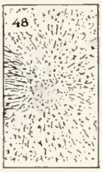
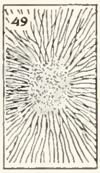
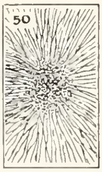
EXPERIMENTAL DATA			DISTRIBUTION OF ASTRAL TYPES AT EACH pH					
			Type 1B	2B	1D	2D	2C	3C
pH	c.c. of Normal NaOH added to Boveri's Reagent	Number of cells studied						
2.2	0	20	14	6				
3.9	2	19			15	4		
4.7	4	23					17	6
5.3	6	23					7	13
5.7	7	14					14	

CHART 4. THE EFFECTS OF VARYING THE HYDROGEN ION CONCENTRATION OF BOVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent at various hydrogen ion concentrations. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure; changes occur in both as the pH is varied.

Experiment 8. The Effects of Varying the Temperature of Boveri's Reagent

Eggs from a single egg-set were fixed in two samples of Boveri's picro-acetic fluid, one chilled to 1°C., the other heated to 95°C. These temperatures were of course slightly modified when the eggs and the ever-present sea-water were added, as the latter were at 21° C.

When the reagent is at 1°C., the same two types (1*B* and 2*B*) occur in about the same proportions as when the eggs are fixed under ordinary conditions at room temperature. But at 95°C. all asters have vague rippled rays and disrupted centers (1*D*). In other words, chilling the fixative produces no deviation from the usual condition, but heating it causes changes (Chart 5). The relation between ray structure and central body structure is once more apparent.

Experiment 9. The Effects of Varying the Duration of Fixation with Boveri's Reagent

Eggs were fixed in Boveri's picro-acetic reagent for periods of time varying from one second to six months, as shown in Chart 6. This experiment shows the speed at which the reagent penetrates and fixes the eggs and also the effects of duration of fixation upon astral structure.

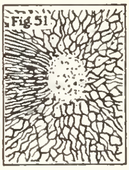
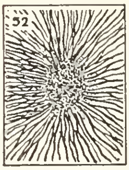
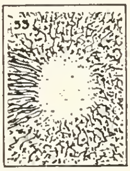
EXPERIMENTAL DATA		DISTRIBUTION of ASTRAL TYPES at each TEMPERATURE		
Temperature of Boveri's Reagent at time of Fixation	Number of cells studied	*Type* 1B  	2B  	1D 
1° C	*31*	*19* *61%*	*12* *39%*	
95° C	*30*			*30* *100%*

CHART 5. THE EFFECTS OF VARYING THE TEMPERATURE OF BOVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent at 1° C. and at 95° C. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure. At low temperatures the central bodies and ray configurations are the same as those occurring at ordinary temperatures; at high temperatures the situation is modified.

The experimental set-up employed when fixing eggs for periods as brief as one or several seconds was as follows: in order to effect instantaneous mixing, two drops of a thick egg suspension were suddenly squirted into 2 cc. of the reagent, which was kept in motion in a small

Stender dish. This proportion of eggs to reagent weakens the fixative so slightly that any effects of dilution can safely be ignored. After the desired interval of fixation, one or more seconds, the entire mixture was poured into 400 cc. of 70 per cent alcohol, the fluid always used to wash material fixed with a picro-acetic reagent. The addition of 2 cc. of eggs and fixative to 400 cc. of alcohol dilutes the fixative so effectively as to stop its further action. However, to make sure that the alcohol itself, plus the limited amount of reagent added with the eggs, did not modify the coagulation product, a control experiment was run, in which eggs were fixed in 70 per cent alcohol containing 0.5 per cent picro-acetic reagent. The asters in these eggs show only vague rays, and central areas entirely undifferentiated from the peripheral part (*2D*, Fig. 57). Hence the washing in alcohol can be dismissed as a factor in producing the types of astral structure occurring in the experiment. The set-up for fixing eggs for longer periods of time was the usual one.[12]

This experiment shows, first, that complete fixation of the eggs in full strength picro-acetic reagent occurs within one second; (the speed of fixation in dilute reagents is not known). In the second place, it shows that different lengths of exposure to the reagent modify the types of central bodies present. These differences, at the various periods of fixation, are as follows:

Fixation time 1, 3, or 6 seconds: All asters have medium coarse undulating rays; 28 of the 34 centers studied are even (*2B*); the remaining 6 are dense (*3B*).

Fixation time 30 seconds: 35 per cent of the asters have disrupted centers and medium coarse rippled rays (*1B*), and 65 per cent have even centers and medium coarse undulating rays (*2B*). These are the same types which occur after the usual periods of fixation for hours, but the proportions in which they occur are intermediate between those of eggs fixed but a few seconds, when there are no disrupted centers, and those occurring under usual conditions, when there is a high percentage of disrupted centers.

Fixation time 10 minutes or fifteen hours: 90 per cent of the asters have disrupted centers (*1B*), and 10 per cent have even ones (*2B*).

Fixation time thirteen days: only 28 per cent of these eggs are of the disrupted type, 72 per cent having evenly filled centers. The percentages of both types occurring in this set are again intermediate, this

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[12] The samples of eggs at the three shortest intervals are intentionally small, because of the effort to keep the egg mass as small as possible. The equally small samples reported for the sets fixed at longer intervals are explained by the fact that this particular batch was fixed a little too early, due to a miscalculation, and but few of the eggs had reached metaphase. However, in spite of the small size of the samples and the error necessarily involved, the major facts are clear.

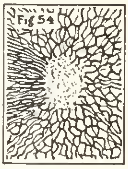
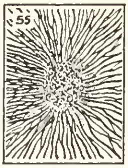
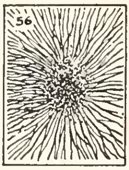
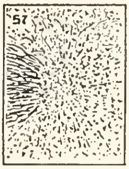
EXPERIMENTAL DATA		DISTRIBUTION OF ASTRAL TYPES AT EACH DURATION of FIXATION			
Duration of fixation in Boveri's Reagent	Number of Cells studied	Type 1B 	2B 	3B 	2D 
1 second.	15		11 73%	4 27%	
3 seconds.	10		9 90%	1 10%	
6 seconds.	9		8 89%	1 11%	
30 seconds.	20	7 35%	13 65%		
10 minutes.	20	18 90%	2 10%		
15 hours.	20	18 90%	2 10%		
13 days.	18	5 28%	13 72%		
6 months.	21		21 100%		
CONTROL:- Eggs fixed in 70% Alcohol containing 0.5% Boveri's Reagent.	20				20 100%

CHART 6. THE EFFECTS OF VARYING THE DURATION OF FIXATION WITH BOVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent for various periods of time. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure. Fixation for a few seconds produces only even central bodies and undulating rays; fixation for hours produces in most cases disrupted centers and rippled rays; fixation for months produces the same result as fixation for seconds.

time between those of eggs fixed for hours and those fixed for months.

Fixation time six months: all asters have even centers and undulating rays (*2B*).

In brief, this experiment shows that fixation for a few seconds produces only asters with filled centers, even or dense, and undulating rays; fixation for minutes and hours produces a majority of asters with disrupted centers and rippled rays and a minority with filled centers and undulating rays; and fixation for months returns the aster to the same condition as that produced by fixation for a few seconds. The major result established in the previous experiments is reaffirmed here: ray structure and central body structure are related.

In all of the experiments reported in this paper except this one, the eggs were left in the reagent from six to fifteen hours. This period of fixation falls within the limits of the groups fixed in this experiment at ten minutes and fifteen hours, during which time no modification of central body structure occurred as a result of the length of exposure to the reagent. Hence it can be assumed that they were not affected by differences in length of exposure to the fixative.

IV. Discussion

All of the astral types previously described are arranged in Chart 7, where they are classified on a three-fold basis:

(A) According to differences in structure there are five classes of central bodies:

Number and Name	*Structure*
1. Disrupted.	Not filled. Entirely empty or containing scattered material.
2. Even.	Filled. Not demarked from ray area. Stained like ray area.
3. Dense.	Filled. Not demarked from ray area. Stained darker than ray area.
4. Slightly demarked, without centriole.	Filled. Slightly demarked from ray area. Stained darker than ray area.
5. Slightly demarked, with single or double centriole.	Filled. Slightly demarked from ray area. Stained darker than ray area.

(B) In shape, the rays take two forms: first, rippled or serpentine, and second, undulating or almost straight.[13] Members of the intermediate, or slightly rippled class are included with the other two (p. 156).

(C) On the basis of coarseness there are five classes of rays: (1) very coarse, (2) medium coarse, (3) delicate, (4) vague, and (5) absent.

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[13] See footnote 4, p. 155.

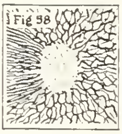
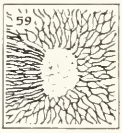
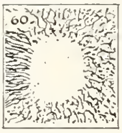
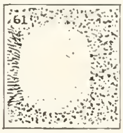
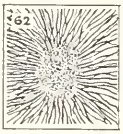
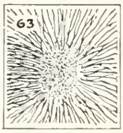
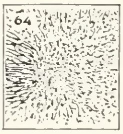
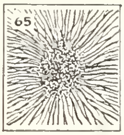
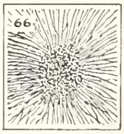
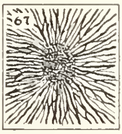
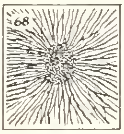
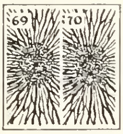
CLASSES OF CENTRAL BODIES		CLASSES OF RAYS A Very Coarse	 B Med. Coarse	 C Delicate	 D Vague	 E Absent
1 Disrupted Empty or Scattered	*Rippled (serpentine)*		 Fig 58 *Type* 1B	 59 1C	 60 1D	 61 1E
2 Even *(not demarked)*	*Undulating (almost straight)*		 62 2B	 63 2C	 64 2D	
3 Dense *(not demarked)*			 65 3B	 66 3C		
4 Slightly Demarked Without Centriole		 67 4A	 68 4B			
5 Slightly Demarked With Centriole single or double		 69 70 5A				

CHART 7. THE ASTRAL TYPES OCCURRING AFTER PICRO-ACETIC FIXATION

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with different modifications of a picro-acetic reagent and various changes in the slide-making procedure. *Result:* Central body structure is related to ray structure. If rays are rippled, centers are disrupted; if rays are undulating, but delicate or medium coarse, centers are usually even (or dense) centrosomes, undemarked from the ray area; only if rays are undulating and also very coarse is there a vaguely demarked centrosome containing a centriole, single or double. These occur in 58 per cent of the cases, and they vary in size and shape

The following facts are apparently established by this study, in metaphase first-cleavage figures of *Chaetopterus* eggs:

I. The Astral Center is Very Unstable.—The chromosomes maintain their usual form under many modifications of technique, except under a few conditions such as extreme dilution of the reagent, or change in the pH. The spindle, too, is modified only occasionally, and its fibers are affected much less than the astral rays. The aster as a whole, although varying in size, is usually clearly demonstrated. The detailed structure of center and rays, however, shows maximum instability.

II. The Variations in the Structure of the Astral Center are Related to Variations in Ray Structure.—(1) If the ray area is completely non-radial or shows but a vague radial organization, the center is either disrupted (1*D* and 1*E*) or undifferentiated from the ray region (2*D*). (2) If the rays are fixed distinctly and are rippled in shape, the center is never filled; it is always disrupted (empty or scattered), regardless of whether rays are delicate (1*C*) or medium coarse (1*B*). These asters, whose centers are either entirely undifferentiated from the ray area or broken and disrupted, will not be discussed further at this time.

(3) If the rays are fixed distinctly and are undulating in shape, the center is always filled, its detailed structure varying with the coarseness of the rays in several respects: (*a*) Size: the coarser the rays, the smaller is the center. When rays are delicate or medium coarse most centers are about $5 \times 6\,\mu$ in size; when rays are very coarse centers are about $4 \times 5\,\mu$ in size. The latter frequently contain minute centrioles. (*b*) Depth of stain: the coarser the rays, the more darkly does the center stain. When rays are delicate or medium coarse, most centers are even, and stain like the ray area (2*B* and 2*C*); occasionally they are dense, staining somewhat more deeply (3*B*, 3*C*, 4*B*). But when rays are very coarse the center always stains darker than the ray area.[14] (*c*) Demarcation from ray area: the coarser the rays, the greater is the tendency for the center to be demarked from the ray region. When rays are delicate or medium coarse, centers are usually not demarked from the ray area, but gradually merge with it (*2B, 2C,* 3*B*, and 3*C*); but when rays are very coarse the center (centrosome) is usually vaguely demarked from the ray region (4*A* and 5A). The degree of demarcation varies not only from center to center, but in

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[14] The difference in appearance between the even (2*B* and 2*C*) and the dense (3*B* and 3*C*) types of centers may be due to nothing but slight differences in the depth of stain. This factor, however, does not account for the dark stain of the centers (centrosomes) in asters having very coarse rays (4*A* and 5*A*). These are deeply stained even when the slide is stained very lightly; in fact such slides must be lightly stained, or the centers are clogged with dye.

different parts of the same center; there is no suggestion of a limiting membrane. (*d*) Presence of centriole: only when rays are very coarse does the center show a double zone of differentiation. When rays are delicate or medium coarse, and also in 42 per cent of the asters with very coarse rays, the center is comprised of but a single zone, the centrosome (*2B, 2C, 3B, 3C, 4A* and *4B*). But in 58 per cent of the asters with very coarse rays the center has two zones of differentiation, the centrosome and the centriole (*5A*).

In short, the center behaves more like an area of variable size and form than like a small individualized body of definite size. The coarser and straighter the rays are, the more condensed is the center—*i.e.,* it is smaller, darker, and more delimited. The twelve central body types occurring in the study constitute a graded series, from a completely empty area at one extreme, to a minute centriole surrounded by a vaguely demarked centrosome at the other.

III. When Centrioles are Present They Show Variations in Structure.—Of the 207 asters studied which have very coarse undulating rays and vaguely demarked centrosomes (*4A* and *5A*: Chart 2, Figs. 11 to 13, representing 187 cases; Chart 3, Fig. 35, representing 20 cases), only 121, or 58 per cent, have centrioles. These have rough and irregular edges and they vary in shape and size. Their configuration in the large aster bears no relation to that of the small one of the same mitotic figure.

IV. The Centriole can be Demonstrated Only under Very Narrow Conditions of Fixation.—To show a centriole when using a picro-acetic mixture, the picric acid must be present in amounts between about 0.01 per cent and 0.2 per cent by weight and the acetic between about 0.05 per cent and 0.4 per cent; all other concentrations used in the present study produce centers without centrioles and rays which are never coarse and undulating. When either or both of the acids are present in very small amounts (Figs. 16, 25, and 42, Chart 3) it is understandable that the reagent may fail properly to coagulate the aster and may thus give meaningless results; but once a concentration is reached which produces very coarse undulating rays and a centriole, it is reasonable to expect that some increase in the amount of either acid would still produce the same kind of coagulation product. Instead of this, however, as soon as the concentration of either exceeds that of the critical zone even slightly, both rays and central bodies are modified. The next paper on *Chaetopterus* eggs will show that equally restricted conditions govern the demonstration of centrioles when diverse reagents are used, both full strength and diluted.

Turning now to the interpretation of these data, it is apparent that the centriole in *Chaetopterus* eggs at metaphase of first cleavage does not behave like a typical centriole, such as is frequently found during the spermatogenesis of many species. The demonstration of the former is dependent upon the simultaneous demonstration of a specific type of ray structure; the latter can be demonstrated whether the mitotic figure is present or absent. The former can be shown only if the fixing agents are present within exceedingly narrow limits of concentration, and even then exhibits variations in size and shape; the latter is readily fixed despite considerable variations in the concentrations of the chemical components of diverse reagents, and shows a similar size and shape in practically all cases. If we are dealing with a true centriole in *Chaetopterus* eggs it is necessary to select one of the twelve centers comprising a continuous series, and regard it as " well fixed," assuming that it is a cell component so constituted chemically that it can be coagulated only under certain narrow conditions, and to dismiss the other eleven types, some of which occur on the same slide, as " poorly fixed."

This behavior of the *Chaetopterus* centriole is not unique. A similar relationship has been established between the structure of central bodies and the structure of rays in cytasters, sperm asters, and first-cleavage figures in *Echinarachnius* eggs (Fry, 1928, etc.) Other studies, soon to be published, demonstrate the existence of a close relation between central body morphology and ray structure in different cell types of various species. Naturally the details differ in each case. In these cells the centrioles exhibit an instability of structure and a relation to ray configuration not generally recognized.

Another and quite different interpretation of the nature of centrioles in *Chaetopterus* eggs is suggested by the data of the present investigation. We may be dealing here, not with an individualized body which exists in the living egg, but with a coagulation product of the focal area of astral rays and spindle fibers. The aster is universally recognized as a complex radial system of converging ray and inter-ray materials, in which the inter-ray substances are crowded out near the center. Since this leaves the central area composed largely or entirely of ray materials, differing both chemically and physically from the peripheral region, is it not possible that this focal area may coagulate differently from the outer parts? Furthermore, its size and physical structure might depend upon the way in which the radial organization of the aster is coagulated —*i.e.,* whether the fixed rays are coarse or delicate, undulating or rippled. Fixation changes in the peripheral portion of the aster may be related to changes at the center. In such an event, one or more of the configurations produced at the center as coagulation products of the focalized rays and spindle fibers might be small, dark, and delimited, and thus simulate an individualized body. In other words, the demonstration of a small demarked body at the astral center may not necessarily prove the existence of a centriole.

On the basis of this interpretation, the centriole in metaphase first-cleavage figures of *Chaetopterus* eggs is in all probability a focal coagulation artifact. Further consideration of this possibility will be held in abeyance until more data are available in this and other species. In the meantime, in this and future papers, bodies behaving as do *Chaetopterus* centrioles will be spoken of as " focal bodies," to indicate the fact that their structure has a close relation to the detailed structure of focalized rays and fibers, in contrast to that of a typical centriole, which is independent of the exact configuration of rays and fibers.[15]

Some of the confusion in the central body problem may be caused by the possibility that the minute bodies which occur at astral centers actually may belong to several categories, such as the following:

(1) *Random Granules.*—At the astral center of certain cells there are present, after a given fixation, various granules like those in the cytoplasm, which differ in size, number, and location. In *Chaetopterus* eggs they occur very rarely, in the even type of central body (*2B*; discussion p. 157). In *Asterias* eggs they are abundant during the first maturation division after Bouin's fixation. They are likewise abundant in telophase first-cleavage figures of *Cerebratulus* eggs after picro-acetic fixation. This evidence has not yet been reported. Where such granules occur, their configurations in certain asters simulate centrioles, either single or double. Such granules have occasionally been interpreted as centrioles in the past, but it is a simple matter to prove that such configurations are but members of a series.

(2) *Focal Bodies.*—To this group belong the central bodies in *Chaetopterus* eggs described in the present study, central bodies in *Echin-*

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[15] The fact that the metaphase centriole of *Chaetopterus* eggs, when present, is either single or double, seems to indicate that it is undergoing division. If such is the case, this is conclusive evidence that we are dealing with a self-perpetuating individualized cell component. But this doubling can also be satisfactorily explained in a way which is in harmony with the suggestion that these structures are focal bodies. However, this question cannot be adequately considered in an investigation which confines itself to metaphase. It will be fully discussed in a later report on first cleavage in *Chaetopterus* eggs, in which centrioles are studied from prophase to telophase, after fixation with reagents which demonstrate them with maximum clearness.

arachnius eggs, and in various other cells to be described in later papers. The morphology of focal bodies is closely related to the exact structure of focalized rays and spindle fibers. In the absence of asters, focal bodies may exist at the spindle-tip, if the latter is sharply focused. If it is blunt, however, as in certain anastral polar-body figures, focal bodies are absent. They are never present before the areas of focalized rays or fibers are formed, and they always vanish when such areas disintegrate, although the outer region of the asters and the middle area of the spindle may still persist. Their behavior differs from that of typical centrioles which exist independently of focalized rays and fibers. Future study may show that certain centrioles which have been assumed to be typical, such as in *Chaetopterus* eggs (Mead, 1898, Wilson, 1930), actually are focal bodies. It remains to be proved, however, whether such focal bodies are individualized structures existing in the living cell, and are unusually difficult to demonstrate, or whether they are merely coagulation artifacts of focalized rays and fibers. In any event their behavior differs so markedly from that of typical centrioles that they should not be confused with them.

(3) *Focal Staining Artifacts.*—These phenomena, described long ago by Fischer (1899) and others, are due to the fact that some asters and spindle-tips, after certain types of fixation, hold the dye with great tenacity at the focal area. In such cases continued destaining makes the body progressively smaller, and finally results in its disappearance. At a certain stage in the process, however, there appears to be a centriole, its size depending upon the degree of destaining. In contrast to such a staining artifact, a focal body exists in the coagulation product as a non-radial body at the astral center, which, if large enough, can be seen in sectioned and unstained material; in other forms, where they are smaller, and when unstained are invisible with white light, they can be demonstrated by ultra-violet photography. A typical staining artifact, on the other hand, contains no demonstrable body at the focal area; instead, the rays or fibers reach the center of the area, and progressive destaining changes the appearance of the center in contour, size, and other points. In the focal body, wide differences in the degree of destaining, short of extreme over- or under-staining, have no effect on its size, since it exists as a non-radial structure at the astral center, and holds the dye somewhat differently than does the surrounding radial area. In some cells the situation is complicated by the fact that both phenomena may be involved: there may be minute focal bodies, in addition to which overstaining may produce staining artifacts in the inner zone of surrounding fibers. These points will be developed in later papers.

(4) *Centrioles.*—These are stable structures which, unlike focal bodies, maintain their characteristic size and shape in spite of extensive modifications in the coarseness and shape of the rays and fibers. In some cells they maintain genetic continuity as individualized bodies from one cell cycle to the next, whether the mitotic figure is present or absent, as in *Drosophila* eggs (Wilson and Huettner, 1931) and in *Amphiuma* leucocytes (Pollister, 1932); in other cases they may disappear during interkinesis, but when present they exhibit a stability of structure unlike that of focal bodies. They are readily demonstrated in their characteristic form by a variety of reagents.

(5) *Blepharoplast-centrioles.*—These are found only in motile cells, or in the forerunners of such cells, with the exception of certain atypical ones, such as the aflagellate sperms of *Ascaris,* recently reexamined by Sturdivant (1931). They have been studied most extensively in the spermatogenesis of animals, where they may arise in the primary spermatocyte or earlier; or they may not appear until the spermatid stage. Like the centrioles of non-motile cells, they are exceedingly stable and show their characteristic morphology when fixed by many different reagents; they are unaffected by changes in the physical structure of focalized rays or fibers; and in some species they are present in the entire absence of such areas. In some cells blepharoplast-centrioles are visible in the living condition. These centrioles are ordinarily thought of as in the same class as those of non-motile cells. This may actually be the case, but the fact that the one is a blepharoplast as well as a centriole, while the other is only a centriole, makes it advisable to give them separate terms which suggest this distinction in their behavior.

(6) *Other Categories.*—There are other types of structures which have been called central bodies such as the following: (*a*) *Bi-lobed bodies of sperm.* In the middle-piece of the echinoderm sperm is a bi-lobed structure of chondriosome material which Boveri misinterpreted as centrioles. That such is not the case was long ago shown by various workers.[16] (*b*) *Erythrocyte centrosomes.* Structures are present during the vegetative phase of erythrocytes in various species which have been interpreted as central bodies. Dawson (1932) has shown that such an interpretation is not valid. (*c*) *Diplosomes of epithelial cells.* Bi-lobed structures lying near the outer part of certain epithelial cells have been identified as central bodies. Whether or not such is actually the case remains to be proved.

Centrioles are generally considered to be the most stable and persistent component of the mitotic figure in the animal cell, and are assumed to play some kind of a formative rôle in connection with the origin of asters and spindles, in some cases giving genetic continuity to successive division figures. It is therefore important that we ascertain whether the cell structures we now call centrioles belong to one category or to several, and that we determine the inter-relationships of these classes. Before this can be done, however, the centrioles of many diverse types of cells must be studied, keeping in mind the various modes of behavior, and also the dangers of misinterpreting certain structures that look like typical centrioles but actually may not be so. In the meantime we must be cautious in generalizing from the behavior of one class to that of the others.

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[16] For a discussion of this see Fry, 1929*a*, p. 105.

V. SUMMARY

In earlier studies of *Echinarachnius* eggs the writer reached the conclusion that their central bodies are the coagulated focal area of converging rays and have no existence as individualized structures in the living cell. The present investigation is the first of a group analyzing central body behavior in a variety of cell types in diverse organisms, for the purpose of ascertaining in each case whether the central body behaves like that of *Echinarachnius* eggs or like a typical centriole, such as that found in many spermatocytes.

The present study confines itself to first-cleavage metaphase asters in *Chaetopterus* eggs, because, in preliminary experiments, the writer failed to find the typical centrioles of that egg previously described by Mead, whose observations were later confirmed by Wilson. The effects upon central body structure produced by the following modifications of technique were observed: the formula, pH, and temperature of the reagent, the duration of fixation, the depth of stain, and other points.

Ray structure is readily modified in coarseness; the shape is also affected, the rays being either rippled (serpentine) or undulating (almost straight). Central body structure is closely related to ray structure: if rays are rippled, centers are disrupted; if rays are undulating but delicate or medium coarse, centers are large homogeneous centrosomes, undemarked from the ray area; only if rays are both undulating and very coarse is there a slightly demarked centrosome, which, in 58 per cent of the cases, contains single or double centrioles, which vary in size and shape.

Centrioles can be demonstrated only under a very limited set of chemical conditions. Among the many modifications of the formula employed, the only ones coagulating centrioles and very coarse undulating rays are those in which the reagent was diluted with about 80 parts of water. In these the percentage by weight of the picric acid is between about 0.01 per cent and 0.2 per cent, and of the acetic acid, between about 0.05 per cent and 0.4 per cent. Any deviations from these conditions produce other types of both centers and rays.

If the central body in *Chaetopterus* eggs is a true centriole, it is atypical in several respects: the great instability of the central body area, the close relation between its morphology and the exact configuration of the fixed rays, and the narrowly limited conditions of fixation under which centrioles can be shown.

The suggestion is made that, as in *Echinarachnius* eggs, the various kinds of central bodies occurring may be various types of coagulation products of the focal area of converging rays, the morphology of the center depending upon the way in which the rays are fixed; only by chance do certain of them simulate individualized structures. Further consideration of this point awaits additional data.

Various classes of minute bodies occurring at the centers of fixed asters are discussed, and the suggestion is made that heretofore we have applied the single term centriole to several very diverse classes of structures, which often look alike but actually behave so differently as to warrant a careful consideration of whether or not they belong to several different categories.

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EXPERIMENTAL PRODUCTION OF CHAINS AND ITS GENETIC CONSEQUENCES IN THE CILIATE PROTOZOAN, COLPIDIUM CAMPYLUM (STOKES)

T. M. SONNEBORN[1]

(From the Department of Zoology, Johns Hopkins University, and the Marine Biological Laboratory, Woods Hole, Mass.)

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I. Introduction

In 1925, Édouard and Mme. Chatton showed that chains of individuals could be produced in *Colpidium campylum* and *Colpidium colpoda* by feeding them a certain strain of the bacterium *Bacillus coli* (*"Colibacillus D"*). The present paper deals with the genetic consequences of a similar chain-formation induced in *Colpidium campylum*[2] by a strain of another bacterium (*Micrococcus sp.*, probably *sensibilis*). The present work is in part an independent corroboration of the work of the Chattons, because the author was unacquainted with their work until the essential points in which the two investigations agree had already been established.

In the present work, though not in the work of the Chattons, experimentally produced chains gave rise to a new racial type,—double animals. As a result of this, the question in the foreground of interest in this paper is: What are the genetic consequences of the experimental production of chains? In the treatment of this question, the following matters will be taken up: (1) the experimental determination of chain-formation; (2) the mode of origin of chains and their subsequent development into races of doubles; (3) the persistence and stability of the races of doubles; (4) the existence of diverse biotypes among doubles and their descendants; (5) an examination of the nature of the processes involved in these changes of hereditary characteristics in vegetative reproduction.

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[1] During the performance of most of the experimental work, the author was Fellow of the National Research Council. The author wishes to acknowledge his indebtedness to Professor H. S. Jennings for many important suggestions, particularly concerning preparation of the manuscript; and to Dr. Ruth S. Lynch for critical reading of the manuscript.

[2] Identified through the courtesy of Professor Édouard Chatton.

II. Materials and Methods

The methods employed for the cultivation of large mass cultures of *Colpidium* were given in an earlier paper (Sonneborn, 1930*a*).[3] The basic fluid was an infusion of 1.5 grams rye grains boiled for ten minutes in 100 cc. spring water, filtered and let stand for 24 hours to "ripen." Two hundred cc. of this infusion plus five boiled rye grains were put into a finger bowl 10 cm. in diameter and 4 cm. deep. This was inoculated with 10 to 15 cc. of an old culture of *C. campylum;* within 24 hours it would develop into a flourishing culture and remain so for several days. When the colpidia began to get smaller and paler, usually in two to six days, subcultures were made in the same way. Only large stock cultures were maintained in this way.

Smaller mass cultures were kept in square salt cellars and in Columbia dishes. These were begun with several drops of ripe fluid (without any rye grains) and ten to thirty colpidia; each day more ripe fluid was added until after three to five days the dish was full (about thirty drops). Subcultures were then made in the same way.

In special experiments designed to investigate carefully the factors determining chain-formation, Columbia dish cultures were made in a different way, following, in many respects, the bacteriological technique employed by Raffel (1930) in the cultivation of *Paramecium.* The standard rye infusion, as soon as filtered, was distributed in cotton-plugged test tubes, autoclaved and stored until needed. When needed, a tube was opened over a Bunsen flame and inoculated by means of a platinum needle with a pure culture of either *Achromobacter sp.,* probably *candicans,* or *Micrococcus sp.,* probably *sensibilis,* grown on beef-agar slants. The inoculated culture fluid was then pipetted into Columbia dishes, inside of Petri dishes. These, and the cotton-plugged pipettes, inside of jars, had all been heated for one hour at 150° C. in a hot-air sterilizer. Each pipette was used only once and the top of the Petri dish containing the Columbia dish was raised only enough to admit the pipette with culture fluid and colpidia. The colpidia had previously been washed according to the method of Parpart (1928). After such a culture was set up, it was opened only once for purposes of subculture.

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[3] In that paper, the species was incorrectly called *Colpidium striatum.*

In all the rest of the work, the colpidia were cultured in isolation on ground glass slides containing two concavities. Twelve of these slides were placed on a glass plate raised on glass supports in an inverted 9-inch Petri dish sealed with water at the bottom. The "ripe" rye infusion was used as cultivation medium, one drop to each concavity. Each day one *Colpidium* was placed in such a drop; 24 hours later the drop was again observed, records of reproduction and other matters of interest were made, and one of the colpidia was transferred to a fresh drop of ripe culture fluid. Each day a similar procedure was followed.

A number of other details of procedure in the isolation cultures are important: (1) The possibility of perpetuating, by daily transfers, differences in bacterial flora between different lines was avoided by collecting a small amount of fluid from each 24-hour-old culture drop and using these small drops for inoculating the culture fluid for the next day. Such cross-inoculations were performed daily in some experiments, less frequently in others. (2) The possibility of repeating daily systematic differences in the treatment of the lines compared was avoided by the following methods: (a) within each moist chamber containing 24 lines, each type of animal in a particular set of comparisons was represented by the same number of lines; (b) within each moist chamber the lines were distributed according to a plan whereby the lines of the same type or race were separated from each other and whereby the arrangement in no two moist chambers was the same; (c) the order in which the moist chambers were transferred was systematically changed from day to day. (3) The possibility of personal bias influencing the results was avoided by assigning to each line for daily identification a name that gave no indication of its genetic history. The key to these names was not consulted until the end of the experiment.

Many of the experiments were conducted at room temperature of 20°–25° C., some were run in a constant temperature chamber which only rarely exceeded the range 22°–23° C. The animals used in all the work here reported were descended from one individual and were never known to conjugate, even when efforts were made to make them do so.

III. The Determination of Chain-Formation

As already mentioned, Édouard and Mme. Chatton were able to induce the formation of chains in *Colpidium campylum* and *Colpidium colpoda* by feeding these species a particular strain of *Bacillus coli*. Other species of bacteria, other strains of *Bacillus coli,* and this strain grown on other than a vegetable base did not induce chain-formation in *Colpidium*. These investigators thus demonstrated that the formation of chains in *Colpidium* is determined by a particular strain of bacteria grown on a vegetable base.

This result of the Chattons was confirmed by the present investigation, as will now be set forth. The rye infusion in which chains of colpidia had arisen was plated out on agar (with the help of Dr. Raffel) and the different kinds of bacteria were separately cultivated. Only two kinds of colonies could be distinguished: one yellow and rapidly growing, the other white and slowly growing. These two species were identified for me through the courtesy of Professor William W. Ford of the Department of Bacteriology, School of Hygiene and Public Health, the Johns Hopkins University. The yellow species was identified as belonging to the genus *Micrococcus* (Bergey) and corresponding closely to the species *M. sensibilis;* the white species was identified as belonging to the genus *Achromobacter* (Bergey) and corresponding closely to the species *A. candicans*.

A number of experiments indicated an increase in the frequency of chain-formation when *Micrococcus* predominated in the food supply. In a few cultures some chains were formed in fluid in which *Achromobacter* predominated; in these cultures, however, *Micrococcus* had not been excluded. In all other cultures in which chains were formed, *Micrococcus* had definitely been inoculated into the culture fluid.

Critical experiments, using the rigorous bacteriological methods described above in Section II, were performed to discover the relation of these two species of bacteria to chain-formation. On July 29, eight normal colpidia were washed, according to Parpart's (1928) method, in two steps: five washings were followed by a lapse of five hours, after which five more washings were made. Four of the eight colpidia were washed in autoclaved rye fluid inoculated with *Achromobacter;* the other four in similar fluid inoculated with *Micrococcus*. By July 31, each set of four had multiplied to form eighteen colpidia. The animals in *Micrococcus* fluid were used to establish nine cultures, each consisting of two colpidia in sixteen drops of autoclaved rye fluid inoculated with *Micrococcus*. The animals in *Achromobacter* were used to establish nine similar cultures in *Achromobacter* fluid. Counts of the number of singles and the number of chains in each of these eighteen cultures were made on August 3. As appears in Table I, not one chain was formed among 46,716 colpidia produced in the pure *Achromobacter* fluid, but 51 chains were formed among 11,912 colpidia produced in the pure *Micrococcus* fluid. The production of chains is clearly deter-

TABLE I

Comparison of the percentage of chains formed in cultures fed Micrococcus exclusively, with the percentage formed in cultures fed Achromobacter exclusively

BACTERIUM SUPPLIED		CULTURES									
		1	2	3	4	5	6	7	8	9	Total
Achromobacter	Number of Colpidia	5765	6720	5741	4849	5186	5815	4240	4852	3548	46716
	Number of Chains	0	0	0	0	0	0	0	0	0	0
	Percentage of Chains	0	0	0	0	0	0	0	0	0	0
Micrococcus	Number of Colpidia	1779	1184	1679	1354	1576	1071	1336	1224	1709	11912
	Number of Chains	21	1	1	5	2	0	6	0	15	51
	Percentage of Chains	1.18	0.08	0.06	0.37	0.13	0	0.45	0	0.88	0.43

mined by the presence of the *Micrococcus*. However, only a small proportion of the colpidia subjected to *Micrococcus* form chains. This is in striking contrast to the result reported by the Chattons with "*Colibacillus D*." In their work, all dividing colpidia formed chains in this bacterium. The relatively small proportion of chains produced with *Micrococcus* may have been due to the fact that this bacterium was cultivated on beef agar before being introduced into the rye fluid with *Colpidium*. The Chattons, it will be recalled, showed that the chain-inducing power of *Colibacillus D* was lost when it was grown on other than vegetable bases.

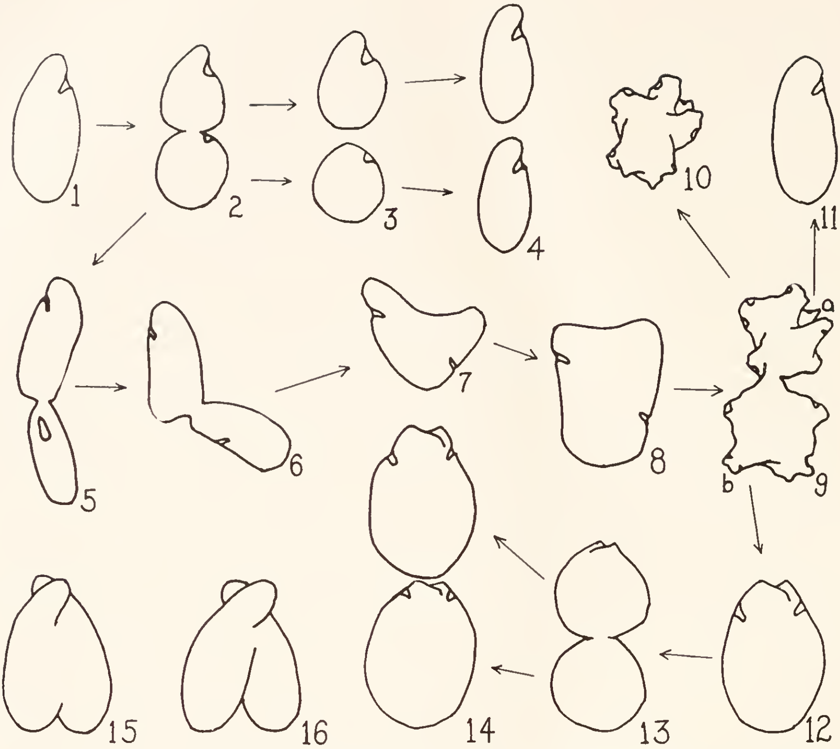
In the present work the existence of additional factors in the determination of chain-formation was indicated by the considerable variation in percentage of chains formed among the nine cultures reared on pure *Micrococcus*. One of these factors may be the concentration of colpidia in the culture fluid. Although all cultures began with two colpidia in sixteen drops, there was much variation in their concentration at the end of the experiment. The mean number of animals per micropipette drop varied from 48.7 to 84.7 colpidia. The five cultures in which there were more than 70 colpidia per droplet produced, on the average, 0.54 per cent chains; the four cultures in which there were less than 60 colpidia per drop produced, on the average, 0.11 per cent chains. There is, therefore, some indication that the percentage of chains formed when fed *Micrococcus* depends to some extent on the concentration of colpidia in the fluid.

The essential point, however, is that the difference between cultures which remain normal and cultures which produce chains is due to the difference between the effects of two diets: cultures fed only *Achromobacter* remain normal; cultures fed *Micrococcus* produce a small percentage of chains. This dependence of chain-formation in *Colpidium* upon the presence of particular strains of bacteria confirms the earlier results of the Chattons. The latter investigators attempted also to induce chain-formation in other genera of ciliates with the strain of bacteria they found effective for *Colpidium*. Such attempts failed completely. The present investigator also attempted to induce chains in *Paramecium aurelia*, by feeding this species the strain of *Micrococcus* found to be effective for *Colpidium*. Like the attempts of the Chattons, this attempt also failed. It appears that *Colpidium* is a genus particularly susceptible to this type of bacterial action.

IV. Origin of Chains and Their Subsequent Development

Chains originate simply as a consequence of the development of the adult form in the two parts of a dividing individual, in the absence of the normal separation of these two parts. A normal adult (Fig. 1) becomes constricted transversely in about the mid-region (Fig. 2). In normal fission, separation of the two parts occurs at this stage (Fig. 3), and each part develops the adult form (Fig. 4). In chain-formation, the two parts attain the adult form while still united (Fig. 5), and separation of the parts is either much delayed or permanently suppressed. In normal fission, the processes of transverse constriction and separation of parts take about twenty minutes; in chain-formation, the two parts remain together several hours or permanently. The parts of the chains usually remained united several hours in the work of the Chattons, but the union was permanent in most, if not all, of the chains produced in the present work. The Chattons also found that some chains developed into three or four parts as a consequence of a second incompleted fission, and that all chains possessed elongated alimentary vacuoles. In the present work, the alimentary vacuoles of chains retained the normal spherical shape and no chains of three or four parts were observed. In all chains isolated for observation, the junction between the two parts widened and their aboral surfaces gradually bent more and more towards each other (Fig. 6). This process continued, with increasing fusion of the two aboral surfaces (Fig. 7), until their entire lengths were completely fused (Fig. 8). Such individuals thus contained two complete sets of internal structures arranged along parallel axes, but with opposite polarity: the former anterior aboral surface of each part was fused to the former posterior aboral surface of the other part. From such heteropolar double animals, multiple monsters of irregular form (Fig. 9) arose as a consequence of further incomplete fissions: sets of internal structures (nuclei, oral grooves, etc.) multiplied, but the new sets did not separate as new individuals. The usual fate of such irregular multiple monsters was either to die or to break up into smaller irregular monsters (Fig. 10). Occasionally, however, the multiple monsters produced apparently normal individuals (Fig. 11). These gave rise to normal descendants. Less frequently, multiple monsters produced double individuals (Fig. 12) with their two sets of internal structures having identical polarity. The identical polarity of the two parts of these doubles is in contrast to the polarity of the doubles (Fig. 8) earlier formed. The production of such homopolar doubles from multiple monsters was not reported by the Chattons.

The probable mode of origin of normals and homopolar doubles from multiple monsters was indicated by the structure of the monsters. Occasionally, a projection on a monster contained a single set of structures (Fig. 9*a*); fission across such a projection would yield an indi-



FIGS. 1–16. The normal division cycle and the origin of doubles from chains and from "pseudo-conjugants." Figs. 1, 4, 5, 6, 7, 8, 11, 12, 13, and 14 from camera lucida drawings; Figs. 2, 3, 15, and 16 from notebook sketches; Figs. 9 and 10 diagrammatic. Figs. 1 and 11, normal adults; Fig. 2, normal fission nearly completed; Fig. 3, fission just completed; Fig. 4, recent products of fission that have already attained adult form; Fig. 5, chains of two parts, each part having the elongated adult form; Fig. 6, chain with the two parts united over a wider region and forming an obtuse angle; Fig. 7, chain with the two parts united over a large part of their aboral surfaces and forming an acute angle; Fig. 8, heteropolar double; Fig. 9, multiple monster containing a projection (*a*) with one set of structures and another projection (*b*) with two sets of structures lying parallel and having the same polarity; Fig. 10, small multiple monster; Fig. 12, homopolar double; Fig. 13, homopolar double undergoing fission; Fig. 14, homopolar doubles produced by fission of a homopolar double; Fig. 15, "pseudo-conjugant" normal singles united laterally except at anterior end; Fig. 16, "pseudo-conjugant" normal singles united laterally in mid-region.

vidual with normal structure. Rarely, a projection (Fig. 9*b*) contained two sets of structures with the same polarity; fission across such a projection would yield a homopolar double.

Homopolar doubles are of particular importance, from the point of view of the present paper, because they gave rise by fissions to biotypes of homopolar doubles, as shown in Figs. 13 and 14. The production of multiple monsters from chains and the formation of homopolar doubles by multiple monsters occurred not only once, but many times. All the homopolar doubles studied gave rise to biotypes of doubles like themselves.

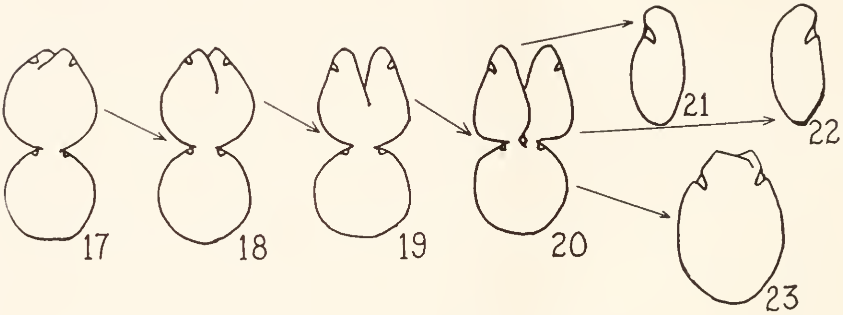
Similar doubles were formed once in an entirely different way. This occurred in a culture of singles left in a Columbia dish a day after most of the colpidia had been removed from it to a finger bowl. (This culture had been fed rye infusion inoculated with *Micrococcus.*) Two individuals (Fig. 15) were found loosely united laterally except at the anterior ends where they did not touch each other; two other individuals were loosely united laterally in the mid-region, but were free at both ends (Fig. 16). These pairs were isolated. Next day, the former had divided into two completely fused pairs; the latter into one completely and one incompletely fused pair. The descendants of each completely fused pair remained united and, like the homopolar doubles produced by multiple monsters, gave rise to biotypes of doubles. These biotypes were kept for three months and, as far as could be judged, could have been kept indefinitely. Thus, in addition to their origin from multiple monsters, biotypes of homopolar doubles arose by lateral fusion of two singles. The superficial resemblance of this fusion to conjugation comes to mind at once, but there are two things that should be remembered in this connection. In the first place, no other phenomenon which might be called normal or abnormal conjugation was ever observed during four years of observation of this species. Secondly, fusions resulting in permanent doubleness are not to be observed in true conjugation. Biotypes of doubles formed by this "pseudo-conjugation" were maintained in mass cultures but not studied farther. In all that follows, it is to be understood that the biotypes of doubles referred to are those that arose from multiple monsters.

V. The Permanence and Stability of Doubles

In both mass and isolation cultures, in both *Achromobacter* and *Micrococcus* fluid, some doubles divided to form two singles and one double instead of two doubles. This came about in the following way. Some doubles (Fig. 17) possessed a small median cleft in the anterior end. In the anterior product of division (Fig. 18) of such an animal, the cleft was usually deeper. Successive anterior products of division (Figs. 19 and 20) contained deeper and deeper clefts until finally one animal (Fig. 20) contained so deep a cleft that the usual transverse fission resulted in the production of two singles (Figs. 21 and 22) from the anterior end, one double (Fig. 23) from the posterior end. The singles and double thus formed reproduced true to their respective types.

This production of singles by doubles raises the question of the permanence of doubles. Will doubles all eventually transform into singles? Or can doubles be maintained indefinitely? As will appear, the answer to these questions depends somewhat on whether mass cultures or isolation cultures are studied. The details of this matter in the two types of cultures will now be set forth.

On April 12, 1930, a mass culture was started of each of eight clones[4] of doubles. These were cultivated in rye infusion inoculated with *Achromobacter,* but the cultures were not kept strictly pure in bacterial content. To each culture fresh fluid was added daily for three or four days, until the original individuals had multiplied to the capacity of the small Columbia dishes. Then the fluid was vigorously sucked up and expelled from a pipette until the animals were uniformly distributed throughout the fluid and a sample of one or two drops was used to start a new culture in fresh culture fluid. At intervals of three or four days the process of renewal was repeated and observations were made on the proportions of doubles and singles present. At the end of 41 days (May 22), after twelve renewals of the cultures, all clones of doubles except one (clone 2) had transformed into populations consisting exclusively of singles.



FIGS. 17–23. Origin of singles from homopolar doubles. Figs. 17, 21, 22, and 23 from camera lucida drawings; Figs. 18, 19, and 20 from notebook sketches. Fig. 17, homopolar double with short median anterior cleft. Fig. 18, double derived from the anterior part of the double shown in Fig. 17; the cleft is deeper. Fig. 19, double derived from anterior part of double shown in Fig. 18; the cleft is still deeper. Fig. 20, double derived from the anterior part of the double shown in Fig. 19; the cleft extends posteriad beyond the fission plane. Figs. 21 and 22, singles derived from anterior part of double shown in Fig. 20. Fig. 23, double derived from posterior part of double shown in Fig. 20.

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[4] The word "clone" is used here in the usual sense of one individual and its vegetative descendants. Arbitrarily, each double produced by a multiple monster has been considered as giving rise to a different clone; this is meant to imply no assumption as to diversity of biotype,—a question treated extensively in Section VI.

A new set of cultures of doubles of these eight clones was started on May 27. In the cultivation of these, the rye infusion was inoculated with *Micrococcus* instead of *Achromobacter*. Renewals of the cultures were made on May 31, June 4, and June 9, before they had to be abandoned. By this time, nine of the cultures had transformed into singles: four of them contained no singles at all, but in the other four 12 per cent to 40 per cent of the colpidia were singles. The average for these four cultures was 26.25 per cent. Comparison with the cultures fed *Achromobacter* after three renewals showed that there were much smaller proportions of singles in the cultures fed *Micrococcus*. Four clones that contained an average of 32.5 per cent singles when fed *Achromobacter* contained no singles when fed *Micrococcus*. The other four cultures averaged 58.75 per cent singles in *Achromobacter*, but only 26.25 per cent singles in *Micrococcus*. This difference in rate of transformation when fed different kinds of bacteria is probably due to a corresponding difference in the rate of multiplication; the colpidia multiplied more rapidly when fed *Achromobacter* than when fed *Micrococcus* (see Table I).

With both types of food, doubles persisted for many generations; hence maintenance of doubleness does not depend on a diet consisting chiefly of *Micrococcus*. However, in both types of food the cultures of doubles gradually contained a larger and larger proportion of singles. In cultures fed *Achromobacter*, all clones (except clone 2) eventually contained only singles. Had the cultures fed *Micrococcus* been observed longer, in all probability they too would have become completely transformed, though this doubtless would have required a longer time than in the cultures fed *Achromobacter*.

It is important to know how this change from doubles to singles took place. Was it a consequence of the death of all doubles or of the transformation of them all into singles? Or was it a consequence of the elimination of doubles in some other way? If the former alternative, then the biotypes of doubles are essentially "Dauermodificationen"; if the latter, they may not be transient biotypes at all. That the latter alternative is the correct one will appear from the records of isolation cultures given below. Meanwhile it is of interest to discover what factors were responsible for the elimination of doubles from mass cultures.

Obviously, an essential factor in this transformation was the production of singles by doubles. If this occurred repeatedly and was not counterbalanced by other factors, it alone would eventually result in the replacement of doubles by singles under the conditions of culti-

TABLE II

Comparison of fission rates of doubles and singles derived from them. Fission rates given in mean number of divisions per line per day.

	PERIOD	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	WEIGHTED MEAN
Clone 3	Number of singles	5	9	11	48	42	39	—
	Means for singles	2.80	2.73	3.11	2.81	3.61	3.59	3.240
	Means for doubles	2.79	2.74	2.90	2.72	3.31	3.33	3.063
	Number of doubles	6	10	12	47	44	45	—
Clone 7	Number of singles	4	—	2	42	39	33	—
	Means for singles	2.69	—	3.00	2.76	3.65	3.41	3.224
	Means for doubles	2.52	—	2.64	2.48	3.11	3.12	2.851
	Number of doubles	6	—	9	46	40	39	—

vation here employed. The doubles fail to double their number at the fissions which yield one double and two singles; but the singles double their number at every fission and the proportion present is continually being increased by the transformation of some doubles into singles. Hence, in the sampling method of culture renewal here employed, eventually too small a proportion of doubles will be present to find a place in the sample. Thus the rate of transformation of a series of cultures would depend on the frequency with which doubles produce singles. This frequency was not ascertained, so that the relative importance of this factor remains unknown.

Other factors, however, become important as soon as some singles have been produced. The subsequent changes in proportion of the two types present must then depend partly on their relative rates of multiplication and partly on their relative rates of mortality.

The fission rates of the singles and doubles of clones 3 and 7 are given in Table II. In clone 3, the singles reproduced 0.1 to 0.3 fissions per day more rapidly than the doubles in all periods except the first two. In these two periods there was practically no difference (0.01 fission per day) between the means for the two groups. However, there were fewer (ten or less) lines in each group during these periods than in any of the later ones; hence, the comparisons during these periods are correspondingly less valuable than during the other periods in which the difference between the two groups was well marked. In clone 7, there can be no doubt that the singles reproduced more rapidly than the doubles. The difference is well marked in all periods for which comparisons are available, even in those in which but few lines were compared. It can be said, therefore, that certainly in clone 7, and very probably in clone 3, the singles reproduced more rapidly than the doubles.

The mortality rates for these groups are given in Table III. With a negligible exception in clone 3, during the period July 4–7, the mortality of singles in both clones 3 and 7 is consistently greater than the mortality of the corresponding doubles. In both clones the mortality rate for the total of all periods is over twice as great among the singles as among the corresponding doubles.

Therefore, in the series of mass cultures of doubles, the gradual change of the proportions of doubles and singles present was not brought about by differential mortality,—indeed was retarded by it; but was due to the continual production of singles by doubles and to the faster reproduction of the singles thus produced. That the death of all doubles or the transformation of them all into singles played no rôle in their extinction is demonstrated by the following account of isolation cultures of doubles.

On April 22, 1930, 48 lines of doubles were isolated in concavities on culture slides. Among these lines eight clones were represented by six lines each. Each line was cultivated in isolation until June 2, 1930. During these 42 days of culture, every clone continued to maintain itself as doubles; no clone died out or transformed completely into singles.

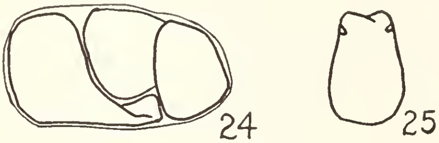
Table III

Comparison of mortality rates of doubles and singles derived from them

		PERIOD	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTALS AND MEANS
Clone 3	Singles	No. line-days	50	60	192	188	230	720
		No. died	0	1	0	6	9	16
		No. deaths per 100 line-days	0.0	1.7	0.0	3.2	3.9	2.2
	Doubles	No. deaths per 100 line-days	0.0	0.0	0.05	2.1	0.9	1.0
		No. died	0	0	1	4	2	7
		No. line-days	50	60	191	190	234	725
Clone 7	Singles	No. line-days	—	52	186	179	218	635
		No. died	—	9	8	8	16	41
		No. deaths per 100 line-days	—	17.3	4.3	4.5	7.3	6.5
	Doubles	No. deaths per 100 line-days	—	3.3	1.1	4.4	3.1	2.9
		No. died	—	2	2	8	7	19
		No. line-days	—	60	188	183	225	656

Singles appeared occasionally along with the doubles, especially during the early history of the lines; but during the last 18 days of cultivation no singles were produced in any of the lines. This change in the frequency with which singles were produced was probably a consequence of the method of selection followed each day, for each day the line was perpetuated by the most perfectly doubled individual present (that is, by the one showing the least development of an anterior cleft). The period (42 days) during which these isolation cultures were maintained as doubles was as long as the period (41 days) during which the mass cultures had transformed completely to singles. Further, these same clones of doubles were maintained from April 5 until October 15. Part of this time they were in isolation culture, part of the time in mass culture; in the latter renewals were made by selecting chiefly doubles. As the usual rate of reproduction was three fissions per day, about 582 generations must have passed while the colpidia remained double. At the end of this time the cultures were discontinued, but there was no reason to suppose that the doubles could not have been maintained indefinitely.

The stability of the doubles was further demonstrated by their maintenance of organization through encystment. The one time encystment was observed in four years of close attention to *Colpidium campylum,* it occurred in a line of double animals, during isolation culture, 34 days (more than 100 generations) after this line originated from a multiple monster. The cyst (Fig. 24) was discovered less than 24 hours after it formed; in it there were three vigorously moving doubles. Of these three, one was large, two small. Within half an hour, the larger animal divided into two. The cyst remained in this condition one more day. On the third day, five animals were present in the cyst; on the fourth day, six animals. On the fifth day, the cyst was opened with a fine glass needle to allow the encysted animals (of which one is shown in Fig. 25) to emerge. Each of these was a double animal; four of them were used to initiate separate lines of descent. Records showed no differences among the emerged animals and their descendants or between the descendants of encysted and non-encysted members of the same clone, so that more detailed studies on this matter were not made. However, the important point is this: in spite of the reorganizations known to occur in cysts of ciliates, the encysted colpidium did not reorganize as a normal single, but emerged from as it had entered the cyst,—with the double organization.



FIGS. 24 and 25. Both from notebook sketches and measurements made with ocular micrometer. Fig. 24, cyst $164\,\mu \times 85\,\mu$ containing three double colpidia, two small and one large. Fig. 25, one of these doubles immediately after excystment.

VI. DIVERSITY OF BIOTYPE AMONG DOUBLES AND AMONG THE SINGLES PRODUCED BY THEM

The origin of diverse biotypes during vegetative reproduction has been the subject of numerous investigations (see review by Jennings, 1929). To these must be added the present one on *Colpidium,* in which it has been shown (Section IV) that biotypes of doubles originate from a clone of singles under the influence of a special environmental condition (diet including *Micrococcus*). Further (see Section V), some individuals in these biotypes of doubles give rise to new biotypes of singles. We take up in this section the question of whether there arise other biotypic diversities among the doubles and the singles produced by them. Of this question there are several aspects: (1) Do biotypic diversities exist among the different clones of doubles? (2) Do biotypic diversities exist among singles derived from different clones of doubles? (3) Do biotypic diversities exist among singles produced independently from the same clone of doubles? (4) Do biotypic diversities exist between singles produced by doubles and singles not descended from doubles? (5) Do biotypic diversities exist among different lines of descent within a clone? These questions will now be taken up in the order mentioned.

TABLE IV

Comparison of fission rates of doubles of clones 3 and 7. Rates given in number of divisions per line per day.

PERIOD		May 1–7	May 8–14	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	Weighted mean for 8 periods
Clone 3	No. lines	6	6	6	10	12	47	44	45	
	Fission rate	3.05	3.05	2.79	2.74	2.90	2.72	3.31	3.33	3.064
Clone 7	Fission rate	2.95	2.73	2.52	2.58	2.64	2.48	3.11	3.12	2.835
	No. lines	6	6	6	10	9	46	40	39	
Clone 3 minus Clone 7		0.10	0.32	0.27	0.16	0.26	0.24	0.20	0.21	0.229

(1) *Biotypic diversities among different clones of doubles.*—Eight clones of doubles were compared in (*a*) the rate of decrease in proportion of doubles present in series of mass cultures; (*b*) the rate of multiplication; and (*c*) the rate of mortality.

(*a*) Striking differences in the rate of decrease in proportion of doubles present appeared among the clones cultivated in series of mass cultures, as described in Section V. In the series fed *Achromobacter,* at the end of the period of observation, no doubles remained in any of the eight clones, except in clone 2. In this clone, however, the vast majority of colpidia present were still doubles. Clearly, the rate of decrease in proportion of doubles was less in clone 2 than in any other clone. This was apparent in both types of food cultures and at all stages in the series. For example, after three renewals of the cultures, all colpidia of clone 2 were still double when fed *Micrococcus* and 95 per cent were double when fed *Achromobacter.* But in clone 3 only 60 per cent of the colpidia were double in *Micrococcus* and only 5 per cent in *Achromobacter.* Between these two extremes, in clone 8, 88 per cent of the colpidia were doubles in *Micrococcus,* 60 per cent in *Achromobacter;* and in clone 1, 72 per cent were doubles in *Micrococcus,* 40 per cent in *Achromobacter.* The different clones thus manifested at least four different rates of transformation and these differences between the clones were the same in both types of culture fluid.

(*b*) An extensive comparison was made of the rates of multiplication in clones 3 and 7. Their mean fission rates for eight periods of from four to seven days each are given in Table IV. In all periods clone 3 multiplied more rapidly than clone 7. At the end of the third period, the slowest line of clone 3 was selected to give rise to all later members of the clone; at the same time, the fastest line in clone 7 was selected to give rise to all later members of this clone. As appears in the table, this radical adverse selection in both clones changed neither the direction nor the magnitude of the difference between the two clones. Clone 3, for the eight periods, had a mean rate of 3.064 fissions per line per day; during the same time, clone 7 had a mean rate of 2.835 fissions per line per day. The different periods gave results very similar to the general mean: in five of the eight periods the excess of clone 3 over clone 7 was between 0.20 and 0.27 fission per line per day; in two periods it was below this range (0.10 and 0.16 fission) and in one period above it (0.32 fission). There can be no doubt of the uniform hereditary difference between clones 3 and 7 in fission rate.

(*c*) The rates of mortality were also extensively compared in these two clones (see Table V). In seven periods, extending over a period of 86 days and including records for 54 of these days, the mortality rates were 0.78 deaths per 100 line-days in clone 3 and 2.40 deaths per 100 line-days in clone 7. In no period is the mortality rate of clone 3 higher than that of clone 7. The difference in mortality rates of these two clones appears constantly through all parts of the experiment and is thus a biotypic diversity.

Among the clones of doubles here compared, clones 2 and 8 arose at different times from one multiple monster and the other six clones arose at different times from another multiple monster. Thus, differences in the rate of decrease in proportion of doubles present in series of mass cultures existed between clones derived from different multiple monsters: clones 2 and 8 as compared with clones 1 and 3; but similar differences also existed between clones derived from the same multiple monster: the rate in clone 2 differing from that in clone 8, and the rate in clone 1 differing from that in clone 3. Likewise, differences between clones of doubles (clones 3 and 7) derived from the same multiple monster were found in rate of fission and in rate of mortality.

Table V

Comparison of mortality rates of doubles of clones 3 and 7

	PERIOD	April 22–May 14	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTALS AND MEANS
Clone 3	No. line-days	120	48	50	60	191	190	234	893
	No. died	0	0	0	0	1	4	2	7
	No. deaths per 100 line-days	0.0	0.0	0.0	0.0	0.05	2.1	0.9	0.78
Clone 7	No. deaths per 100 line-days	1.7	0.0	0.0	3.3	1.10	4.4	3.1	2.40
	No. deaths	2	0	0	2	2	8	7	21
	No. line-days	120	48	50	60	188	183	225	874

(2) *Biotypic diversities among singles derived from different clones of doubles.*—Clones 3 and 7 of doubles have just been shown to differ in rate of fission and in rate of mortality. Do the singles produced by these two clones of doubles differ in the same way? One single from each of these two clones of doubles was permitted to give rise to a number of lines and the mean fission rates of these two groups of singles were compared in four periods. The differences found were small and not constant, so that no significance may be attached to them. In mortality rate, however, the situation was different. As appears in Table VI, in all four periods the rate of mortality is greater—usually very

much greater—among the singles of clone 7 than among the singles of clone 3. The rate for the total time is 2.39 deaths per 100 line-days in clone 3 and 6.33 deaths per 100 line-days in clone 7. The rate for clone 7 is thus 2.65 times as great as that for clone 3. In connection with this difference, it is of interest to note (see Table V) that the doubles of clone 7 had a mortality rate 3.08 times as great as the doubles of clone 3.

Table VI

Comparison of mortality rates of stocks of singles produced by one single from a clone 3 double and one single from a clone 7 double

	PERIOD	June 4–11	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTAL
Singles from clone 3 double	No. of line-days	42	60	192	188	230	712
	No. of deaths	1	1	0	6	9	17
	No. of deaths per 100 line-days	2.4	1.7	0	3.2	3.9	2.39
Singles from clone 7 double	No. of line-days	44	52	186	179	218	679
	No. of deaths	2	9	8	8	16	43
	No. of deaths per 100 line-days	4.5	17.3	4.3	4.5	7.3	6.33
Rate of clone 7 singles minus rate of clone 3 singles		2.1	15.6	4.3	1.3	3.4	3.94

(3) *Biotypic diversities among singles produced independently from the same clone of doubles.*—As set forth above, singles were produced two at a time from the anterior half of a double animal. One arises on the right side, one on the left (see Fig. 20). Do the two singles of such a pair differ? That there might be a difference in symmetry was suggested to me by Mr. Donald Costello; but I was unable to detect it. However, it was clear that the two singles of a pair differed sometimes, but by no means always, in other respects. These differences occurred when the anterior cleft of the parental double was much shifted from the usual median position. Frequently the narrower part was separated off as a single one division or more before the broader part. The single produced from the narrower part was invariably narrow, pale, and short as compared with the single produced from the broader part or with the singles ordinarily produced from doubles. The fates

of these two different types of singles were frequently observed. The difference in their fates is illustrated by an experiment on 48 lines observed from May 16–19, 1930. In this group, of the seven singles derived from the narrower parts of unequally cleft doubles, the progeny of four died. Among the 41 lines not derived from narrow parts of unequally cleft doubles, the progeny of only two died. Thus 57 per cent of the one group died as compared with only 5 per cent of the other group. Many other observations confirmed the results in these groups, so that there was no doubt of the very much greater mortality among the descendants of singles derived from the narrower parts of unequally cleft doubles. Whether the singles produced from the ordinary medially cleft doubles at one time were diverse from those produced from a double of the same clone at another time was not investigated.

TABLE VII

Comparison of mortality rates of a group of singles descended from one single produced by a double of clone 7 and one single not descended from doubles.

	PERIOD	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTAL
Singles not descended from doubles	Number of line-days	479	384	376	473	1712
	Number of deaths	5	3	10	8	26
	Number of deaths per 100 line-days	1.0	0.8	2.7	1.7	1.52
Singles from clone 7 double	Number of line-days	52	186	179	218	635
	Number of deaths	9	8	8	16	41
	Number of deaths per 100 line-days	17.3	4.3	4.5	7.3	6.46
Singles of clone 7 minus singles not descended from doubles		16.3	3.5	1.8	5.6	4.94

(4) *Biotypic diversities between singles descended from doubles and singles not descended from doubles.*—Comparisons were made between singles descended from doubles and singles not descended from doubles in rate of fission and in rate of mortality. The differences found in rate of fission were small and not consistent, so that no significance may be attached to them. In rate of mortality, however, the differences were clear (see Table VII). The total difference between the singles not de-

scended from doubles and a group of singles descended from one single produced by a double of clone 7 is very great: the mortality rate of the latter group is 4.25 times as great as that of the former. This difference is clearly manifested in every period and demonstrates a biotypic difference in rate of mortality between these two groups of singles.

(5) *Biotypic diversities within a clone.*—As already set forth, the most striking differentiation into biotypes that occurs within a clone of doubles is its splitting into biotypes of singles and doubles. This occurred repeatedly in all clones of doubles studied. Furthermore, the biotypes of singles so formed within a clone of doubles were not all alike: some were normal singles, others were narrow, pale, and highly inviable. In addition to these biotypic differences, there were indications of other biotypic differences within a clone, among the doubles themselves. In the isolation cultures of doubles maintained from April 22 until June 2, the frequency with which singles were produced changed strikingly. This was probably brought to light by the practice of selecting daily from among the individuals produced during the previous 24 hours in each line, the individual which showed least development of an anterior cleft. In each line this individual was used to perpetuate the line and the remaining individuals were discarded. In spite of this method of selection, cleft individuals continued to appear and give rise to singles during the early history of these isolation lines. During April 22–30, singles were produced in 31 of the 48 lines of doubles under cultivation. In some lines they appeared more than once. The product of the number of lines in which they appeared by the number of days on which they appeared gives a measure of their frequency of production. This product was 63 line-days, yielding an interval of 6.86 line-days between successive productions of singles. These figures are in striking contrast to those obtained for the same lines during the period May 16–June 2. Not one single was produced during these 864 line-days. In order to bring to light such a great change in the frequency with which doubles produced singles within the same lines of descent, there would have to be genetic differences in frequency of single production among the doubles of each clone. Such a conclusion seems required by the evidence.

Attempts to isolate by selection biotypic differences in rate of fission within clones of doubles, within clones of singles, and within clones of singles descended from doubles were all fruitless. The coefficient of variation of fission rate of a clone of doubles (8.38 per cent) was greater than the corresponding coefficients for singles descended from this clone of doubles (6.46 per cent) and for a clone of singles not descended from doubles (6.66 per cent). But this greater variability of the clone of doubles was probably not an index of the existence of biotypic diversities in fission rate within this clone, because extreme selection did not result in the isolation of sub-clones with diverse fission rates.

VII. Discussion

The racial effects of environmental conditions found here in *Colpidium* bear a striking parallel in many respects to the relations previously found in the rhabdocoel turbellarian, *Stenostomum incaudatum* (Sonneborn, 1930*b*). As in *Colpidium*, so in *Stenostomum*, special environmental conditions induced abnormalities in reproduction resulting in the formation of irregular monsters. These, likewise, gave rise to double animals that differed from each other and produced races of doubles differing in the same way. Further, in both *Colpidium* and *Stenostomum* the races of doubles maintained their character after removal from the environment that induced their formation, except that singles were formed whenever a cleft of sufficient extent occurred in the growing region perpendicular to the plane of fission. In both, singles gave rise to races of singles of higher viability than the doubles from which they arose.

The degree of similarity between a protozoan and a flatworm in the effects of environmental conditions on their hereditary characteristics is particularly striking in contrast to the very different results of most similar work on higher organisms. What is the basis of this difference? It seems to be the method of reproduction. In sexual reproduction, change of hereditary characters depends largely on changes in the nature or in the composition of the chromatin. Environmental conditions of special penetrability are required to get at this material, so that but few environmental conditions are effective in altering hereditary characteristics. In asexual reproduction, on the other hand, change of hereditary characters may be brought about without in the least affecting the nature or composition of the chromatin; changes in the composition of components of a larger order are also capable of self-perpetuation—that is, are heritable. Examples of this are the rearrangement of parts in homopolar doubles in *Stenostomum* and *Colpidium*. That changes in the chromatin were not involved in these examples was demonstrated by the fact that when individuals were produced from cleft parts of doubles, these were always singles and gave rise to biotypes of singles. The type of hereditary change involved in the production of biotypes of doubles in *Stenostomum* and *Colpidium* is similar to the type involved in the production of stocks of *Drosophila* in which the two X-chromosomes are united or in which translocations, inversions, or reduplications have occurred. All such examples illustrate hereditary changes not due to changes in the nature of the germinal material, but due to changes in the number of units or arrangement of units in the germinal material.

It is remarkable that very diverse environmental conditions acting on such diverse organisms as *Colpidium* and *Stenostomum* should result in similar stable types. It may be that this is another example of the stability of whole multiples, as in polyploids; and that of all the teratological consequences of diverse original stimuli, the whole multiples that result are particularly of the viability requisite for survival and self-perpetuation.

VIII. Summary

In a clone of *Colpidium campylum* (Stokes), a small proportion (less than 1.2 per cent) of the individuals formed chains when cultivated in a rye infusion inoculated with the bacterium *Micrococcus sp.* (probably *sensibilis*), but not when the infusion was inoculated with *Achromobacter sp.* (probably *candicans*). Other factors, one of which possibly was the concentration of colpidia in the culture fluid, affected the proportion of chains formed when the colpidia were fed *Micrococcus*. Chains thus produced went through a series of developments including the formation of heteropolar doubles and multiple monsters, and culminating often in the formation of homopolar doubles of a self-perpetuating, relatively stable sort. Similar biotypes of doubles were also formed once as a result of a " pseudo-conjugation." In clones of homopolar doubles, singles sometimes arose by ordinary transverse fission across a double with a deep median anterior cleft. Consequently, mass cultures begun with doubles eventually contained singles also. As the two types multiplied side by side, the relative proportion of singles gradually increased. When the cultures were regularly renewed by taking a sample of the old culture to start a new one, eventually, after many such renewals, doubles entirely disappeared from the cultures, leaving only singles. The change occurred in both *Achromobacter* and *Micrococcus* fluid, but more rapidly in the former; this was probably due to the more rapid reproduction in that fluid. The change in the mass cultures was not due to dying out of doubles or to the transformation of all of them into singles. In isolation culture, lines of doubles were maintained as long as the period required for doubles to disappear entirely from mass cultures. Furthermore, when doubles were deliberately salvaged at each renewal of culture, they were maintained in cultivation, partly in isolation, partly in mass, for 194 days, during which about 582 generations passed. The disappearance of doubles from mass cultures in 41 days or less must therefore have been due to other factors than the inability of doubles to live and reproduce their kind. One of these factors was a differential rate of fission: the singles produced by the doubles of one clone multiplied 0.373 fissions per line per day more than the doubles of this clone. On the other hand, differential mortality counteracted this to some extent, for the mortality rate of singles was higher than the mortality rate of the doubles that produced them. The gradual disappearance of doubles in series of mass cultures was therefore due partly to their repeated production of singles and partly to their lower fission rate. The persistence of doubles during nearly 600 generations, by the end of which time no evidence of inability to maintain themselves had yet appeared, indicates that the type could have maintained itself indefinitely, even when the bacterium that led to its formation was absent or present in but very small quantities. Further evidence of stability of organization was the passage of a line of doubles through encystment without loss of the double organization.

The question of whether diverse biotypes existed among the experimentally produced doubles and their descendants was extensively investigated. (1) Different clones of doubles differed (*a*) in the rate at which doubles disappeared from series of mass cultures, (*b*) in rate of multiplication, (*c*) in rate of mortality. (2) Singles derived from diverse clones of doubles differed in rate of mortality to about the same extent as the clones of doubles from which they had been derived. (3) There were two very different kinds of singles derived from the same clone of doubles: the usual kind and a rarer kind formed by transverse fission across an unequally cleft double. The singles formed from the narrower anterior part of these were narrower, paler, and shorter than ordinary singles and had a much higher rate of mortality. (4) Singles not descended from doubles had a lower rate of mortality than singles descended from doubles. (5) Within a clone of doubles there were genetic differences in the frequency with which singles were produced, for long-continued selection within lines of doubles brought to light very great changes in this frequency. Attempts to isolate by selection biotypic diversities in rate of fission within clones of doubles and of singles failed, although the coefficient of variation of fission rate was higher for doubles than for singles.

The general picture of the genetic consequences of environmental action in the ciliate protozoan, *Colpidium campylum*, is strikingly similar to the picture in the rhabdocoel turbellarian, *Stenostomum incaudatum*. The similarity in these and the difference of both from the genetic effects of environmental action in higher organisms were ascribed to the method of reproduction. In asexual reproduction hereditary changes may arise without altering the nature of the chromatin; they may be due simply to changes in the number of units or arrangement of units in the self-perpetuating parts. The changes induced in *Colpidium* and *Stenostomum* were of this sort and, in this respect, resemble translocations, inversions, and reduplications in *Drosophila*.

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CHROMOSOMES OF ARTIFICIALLY ACTIVATED EGGS OF URECHIS

ALBERT TYLER

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California and the William G. Kerckhoff Marine Laboratory, Corona del Mar, California)

The eggs of *Urechis* that cleave and develop as a result of activation by dilute sea water have been previously shown (Tyler, 1931*a*) to be those which extrude no polar bodies. It would appear then that the embryos produced by such eggs might be tetraploid, diploid, or haploid, depending upon the behaviour of the chromosomes during the first two nuclear divisions. A cytological investigation of such eggs shows that the embryos are diploid in chromosome number, and that only one maturation division occurs.

The preparations were made by a method used by Karl Bělař and similar to that described by him (1928). It consists of joining and later separating two cover-slips, one containing a drop of eggs and the other a drop of fixing fluid. The eggs are flattened to any desired extent and stick to the cover-slips, which can be handled in the same manner as slides containing sectioned material.

Two types of eggs are produced as a result of activation with dilute sea water (Tyler, 1931*a*). In one type the initial behaviour is identical with that of the normally fertilized eggs, two polar bodies are produced but none of the eggs divide. In the other type the initial behaviour is quite different from that of normally fertilized eggs; no polar bodies are produced but practically all the eggs of this type divide and develop.

In making the cytological preparations of the eggs of the first type, use was made of the fact, previously reported (Tyler, 1931*b*), that an inverse relation exists between the total percentage of activation and the percentage of cleavage. Thus treatments giving 100 per cent activation produce only eggs of the first type which do not divide. For preparations of the type which does not extrude polar bodies, the eggs had to be isolated from dishes containing also unactivated eggs and activated eggs of the first type. These eggs can be readily distinguished at an early stage and can be removed for cytological preparations before the time at which the first polar body appears in the eggs of the first type.

The counts of chromosome number were generally made from polar views of anaphase groups inasmuch as precociously divided chromosomes in metaphase might cause difficulty.

The Eggs That Produce Polar Bodies

The behaviour of the chromosomes in the maturation division of the artificially activated eggs that extrude two polar bodies is identical with that of the normally fertilized eggs. The normal diploid number in *Urechis* is most probably thirty-six chromosomes and the haploid number eighteen. The variability in the chromosome numbers shown in the tables is undoubtedly due to errors in counting. The artificially activated eggs (last section of Table I) show the haploid number of chromosomes at the first maturation division. The second polar division also occurs normally and the egg is left with the haploid number of chromosomes, which form a nucleus and move into the center of the egg. A large monaster then forms at about fifteen minutes after the extrusion of the second polar body and the chromosomes distribute themselves irregularly about the astral rays. The monaster disappears and a vesicular nucleus is formed about ten minutes later. The monaster then reappears about twenty minutes later and a larger and variable number of chromosomes are seen. The monaster may disappear and reappear a third time. This behaviour is essentially similar to that described by Herlant (1918) in the sea-urchin for eggs activated by butyric acid. The failure of the eggs of this type to divide appears then to be due to the failure to form an amphiaster.

Table I

FIRST CLEAVAGE OF NORMAL FERTILIZED EGGS		FIRST POLAR DIVISION OF NORMAL FERTILIZED EGGS		FIRST POLAR DIVISION OF PARTHENOGENETIC EGGS	
Chromosome Number	Number of Groups	Chromosome Number	Number of Groups	Chromosome Number	Number of Groups
33	4	15	3	14	2
34	6	16	2	16	5
35	3	17	8	17	10
36	7	18	8	18	6
37	2	19	1	19	1

The Eggs That Divide

In the eggs of this type the germinal vesicle breaks down and tetrad chromosomes appear at about twenty to twenty-five minutes after treatment. At this time the first polar spindle appears in the control eggs. But no spindle is seen in the eggs of this type and at about ten to twenty minutes later the tetrads each form a small vesicular karyomere. The nucleolus generally persists as such throughout this time and about seventeen or eighteen karyomeres may be seen distributed throughout the egg. Later a single large nucleus is formed apparently by the fusion of the karyomeres. The nucleolus remains intact and is seen within the nucleus. This nucleus is generally about two-thirds of the size of the original germinal vesicle and has a granular appearance similar to that of the cytoplasm. The eggs remain in this condition for about an hour, after which the first cleavage spindle appears.

Table II

Parthenogenetic Eggs

FIRST CLEAVAGE		SECOND CLEAVAGE		THIRD CLEAVAGE	
Chromosome Number	Number of Groups	Chromosome Number	Number of Groups	Chromosome Number	Number of Groups
13	1	32	2	32	5
15	4	33	2	33	7
16	8	34	3	34	6
17	5	35	8	35	4
18	5	36	4	36	5
		38	1	37	2
				38	2
				40	1
		45	1	13	2
		46	1	14	2
		48	2	15	2
		50	1	16	4
		53	2	18	5
		54	1	20	1

An attempt was made to determine whether any division of the chromosomes occurred prior to the first cleavage, and whether fusion of the egg nucleus with a submerged polar body nucleus such as described by Morris (1917) in *Cumingia*, occurred in *Urechis*. The evidence shows that such behaviour does not occur in *Urechis*. It is possible to determine this point with some certainty in *Urechis* inasmuch as the eggs which are to divide remain indented until just before the first cleavage. The indented eggs were therefore preserved at close intervals up to that time. No division figures or fusion of two nuclei were observed. Furthermore, the chromosomes on the first cleavage spindle have the appearance of tetrads and are eighteen in number. In anaphase they open out as typical dyads.

It appears then that the first cleavage spindle is identical with the first polar spindle as far as the chromosomes are concerned, and the first division may be considered a maturation division.

At the second cleavage the diploid number of chromosomes is usually seen. This is the case with the twenty anaphase chromosome groups of the five eggs listed in Table II. The chromosomes of these groups occur in more or less closely associated pairs. This means that the chromosomes had divided previous to this division. In other words the second cleavage is a mitotic rather than maturation and the diploid number of chromosomes is retained. In two eggs listed in Table II eight anaphase groups gave chromosome counts approximating fifty-four, the triploid number. The origin of such chromosome groups was not determined, and they were not encountered in the slides of the later stages.

Table III

Parthenogentic Eggs

FOURTH CLEAVAGE		EMBRYOS	
Chromosome Number	Number of Groups	Chromosome Number	Number of Groups
31	2	28	2
32	6	29	1
33	8	30	4
34	7	32	6
35	6	33	3
36	10	34	7
37	4	35	2
38	3	36	3
39	1	38	2
40	1		

In the anaphase of the third cleavage the diploid number is again usually seen. Two eggs were obtained, however, in which the chromosomes were of the haploid number. These must have arisen by the occurrence of both maturation divisions in the first two cleavages of the egg. The haploid number was not obtained again in the later stages of other eggs studied.

The chromosome counts at the fourth cleavage of the egg, Table III, again approximated thirty-six, the diploid number. For the embryos, normal top-swimmers were isolated and preserved in the usual manner. Counts on fifteen pairs of anaphase groups (Table III) showed that the diploid number is present.

The failure of the artificially activated egg that extrudes polar bodies to divide may be attributed to its retaining only the haploid number of chromosomes or to the possession of only the inner central body of the second polar spindle which is incapable of forming an amphiaster. The former is an unlikely assumption inasmuch as some haploid cleavage has been obtained in *Urechis*. But if the interpretation is based upon the behaviour of the central bodies,[1] it is difficult to see why the cleavage of those eggs that produce no polar bodies should go beyond the four-cell stage. At this stage the centrosomes should be equivalent to the three that would have gone into the polar bodies and the one that remains in the egg. It might appear then that when the polar body central bodies come to lie within the egg cytoplasm they are capable of forming amphiasters. It may be pointed out in this connection that the first polar body in *Urechis* may or may not divide. Correspondingly in the artificially activated eggs, one of the cells of the two-cell stage often fails to divide. Similarly in the four-cell stage one of the cells often fails to divide corresponding to the egg cell that receives the inner central body of the second polar spindle. This again may be interpreted to mean that the first polar spindle is used for the first division. However, in a large number of cases all four cells divide, and since no accessory asters have been observed in these eggs, it appears that the inner central body of the second polar spindle has regained the ability to form an amphiaster.

Summary

1. The embryos resulting from the artificial activation of *Urechis* eggs are diploid in chromosome number.
2. The diploid number is apparently obtained by the utilization of the first polar spindle for the first cleavage and the substitution of a mitotic division for the second maturation division.

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[1] This discussion is based on the assumption of the genetic continuity of the centrosomes to which there no longer appears to be serious objection.

Tyler, A., 1931*a*. The Production of Normal Embryos by Artificial Parthenogenesis in the Echiuroid, Urechis. *Biol. Bull.*, **60**: 187.

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PRODUCTION OF CLEAVAGE BY SUPPRESSION OF THE POLAR BODIES IN ARTIFICIALLY ACTIVATED EGGS OF URECHIS

ALBERT TYLER

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California, and the William G. Kerckhoff Marine Laboratory, Corona del Mar, California)

It was suggested in an earlier paper (Tyler, 1931*b*) that those eggs which do not ordinarily divide as a result of artificial activation could be made to do so by suppression of the polar bodies. This was based on the fact that upon activation by means of dilute sea water only those eggs divide that extrude no polar bodies. The other type of egg produced by artificial activation behaves very much like the normally fertilized egg in its initial reactions to the treatment, extrudes two polar bodies, but does not divide. The results of the experiments reported here show that the polar bodies can be suppressed in such eggs by means of a second treatment with dilute sea water and that the eggs then divide.

When *Urechis* eggs are treated with dilute sea water the percentage of the activated eggs that divide after various lengths of exposure bears an inverse relation to the total percentage of activation (Tyler, 1931*b*). Thus exposures resulting in 100 per cent activation give no cleavage, and the eggs are all of the type that extrudes both polar bodies. This simplifies the task of re-treating such eggs, since no unactivated eggs and no eggs of the type that divides are present in the dishes. The percentage of activation can be determined at about ten minutes after the initial treatment, and since the first polar body appears at thirty minutes at room temperature there is ample time for the second treatment.

The first attempts at polar body suppression were made by means of anesthetics such as ether, phenyl urethane, and chloretone in various concentrations. Low temperature was later tried, as was also hypertonic sea water. These agents gave variable results, and in general although the polar bodies were suppressed while the eggs remained under treatment they often appeared later when the eggs were removed to normal sea water.

Dilute sea water was then tried and this was found to be quite an effective agent for suppressing the polar bodies and producing cleavage.

The concentrations used were 50 and 55 per cent sea water. Higher concentrations generally failed to suppress the polar bodies and lower concentrations appeared to injure the eggs.

The Second Treatment with Dilute Sea Water

In these experiments the eggs were first treated for various lengths of time with 30 or 40 per cent sea water. The length of exposure resulting in 100 per cent activation is known fairly well from previous experiments, and so treatments ranging about the optimum time were used. The dishes were then examined to determine which actually showed 100 per cent activation. Large samples of eggs were then transferred from such dishes at various times after the beginning of the first treatment to 50 or 55 per cent sea water. The eggs were usually exposed to the second treatment for 5 to 40 minutes. They were examined in the dilute sea water and later in normal sea water to determine whether or not the polar bodies were suppressed by the treatment. The usual precautions in regard to the amount of water transferred with the eggs, etc. were taken.

Table I

Re-Treatment with 55 per cent sea water. Eggs first treated for 2 minutes with 30 per cent sea water gave 100 per cent activation and all eggs later showed two polar bodies and no cleavage. First polar body out at 30 minutes; second at 40 minutes. *p.b.* = polar body.

Time after first treatment	Length of second treatment	Cleaved			Uncleaved		
		0 p. b.	1 p. b.	2 p. b.	0 p. b.	1 p. b.	2 p. b.
minutes	*minutes*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
25	5	0	0	0	0	0	100
25	10	2	3	0	0	2	89
25	15	20	6	0.2	2	3	69
25	20	36	2	0	34	12	16
25	25	71	2	0	18	9	0
25	30	94	1	0	3	1	1
25	35	96	2	0	1	1	0
25	40	88	0	0.1	7	3	2
40	5 to 40	0	0	0	0	0	100
55	5 to 40	0	0	0	0	0	100

The results of one such series of experiments are given in Table I. The eggs were first treated with 30 per cent sea water and an exposure of two minutes was found to give 100 per cent activation. All of the eggs in this two-minute dish later showed two polar bodies. The first polar body appeared at 30 minutes and the second at 40 minutes after the first treatment. A sample of eggs was transferred to 55 per cent at 25 minutes after the first treatment; that is, 5 minutes before the first polar body was due to appear, and treated for various lengths of time. About an hour later the percentage of cleavage was determined and also the presence or absence of polar bodies. As shown in the table, when the eggs are exposed for 5 minutes to the second treatment with dilute sea water the polar bodies appear and no cleavage is obtained as in the controls. However, upon longer exposures fewer of the eggs show two polar bodies, and after exposure of 25 minutes or more practically none of the eggs show two polar bodies. At the same time the percentage of cleavage increases from zero to 98 per cent. The great majority of the cleaved eggs have no polar bodies. A small percentage of the divided eggs have one polar body (column 4 in the table) and a very few of the divided eggs show two polar bodies.

When the second treatment is applied at 40 or at 55 minutes after the first treatment (*i.e.*, after extrusion of the second polar body) the results are the same as for the control eggs—none of the eggs divide.

Fourteen series of experiments of the type illustrated by Table I were run and all gave similar results. Cleavage was obtained when the second treatment was applied before the time of extrusion of the first polar body and was continued until after the time of extrusion of the second polar body. When the eggs were given equivalent treatments at any time after the second polar body had appeared no cleavage was obtained. Cleavage was sometimes obtained when the treatment was applied after the extrusion of the first polar body. Eggs were also isolated after the second treatment according to the number of polar bodies they showed, and of 200 eggs examined cleavage was obtained in 90 per cent of the eggs that showed no polar bodies, 15 per cent of the eggs with one polar body, and none of the eggs with two polar bodies.

At the first cleavage the doubly treated eggs divided into two or three cells. Of 400 eggs on which counts were made 65 per cent divided into two cells and 35 per cent into three cells. Cleavage often stopped in the four-cell stage. Large numbers of abnormal top and bottom-swimmers but no normal embryos were obtained from the re-treated eggs.

It is evident then that when the polar bodies are suppressed by means of a second treatment the eggs are then capable of division. Suppression of one polar body appears to be less effective in this regard than suppression of both.

Cytological preparations were made of the doubly treated eggs according to the method previously described (Tyler, 1932) in order to determine the behaviour of the chromosomes and centrosomes. Eggs were removed for preservation directly from the dilute sea water and also after their return to normal sea water. In the eggs preserved within twenty minutes after the application of the second treatment the achromatic figure was generally not visible, and the chromosomes appeared as condensed bodies, similar to their metaphase condition. They formed a single group at the pole of the egg. In eggs removed at later times from the dilute sea water the chromosomes were often found in two groups of about 12 to 18 each although generally they appeared in one group of about 18 scattered about in the polar region. When the eggs were returned to normal sea water at 30 minutes after the second treatment and later preserved, they first showed two chromosome groups which were generally associated with two separate asters. At later stages the eggs showed a single group of chromosomes, presumably due to the fusion of the two separate groups. The asters are usually not visible at this time. At the time of cleavage an amphiaster develops, and the chromosomes are seen distributed irregularly about the spindle. The first cleavage divides the chromosomes irregularly and counts of anaphase groups ranged from 8 to 40, the two groups sometimes containing equal numbers and at other times radically different numbers of chromosomes. Later stages were not followed.

The examination of the cytological preparations shows that when the polar bodies are suppressed, the chromosomes first separate into two groups which later come together and distribute themselves more or less irregularly about the first cleavage spindle.

Discussion

An important question involved in the cleavage of artificially activated eggs concerns the origin of the amphiaster. The parthenogenesis experiments of Herlant (1918), Fry (1925), and others on the echinoderm egg are generally taken to mean that central bodies and asters may arise *de novo* and either combine or divide to form an amphiaster. Although this argues against Boveri's view of the genetic continuity of the central bodies, more evidence has recently been presented in its favor from other sources (Sturdivant, 1931; Wilson and Huettner, 1931; Pollister, 1930; and Johnson, 1931). The parthenogenesis experiments on *Cumingia* (Morris, 1917; Heilbrunn, 1925) and on *Urechis* (Tyler, 1931*a*) show that cleavage is obtained when the eggs fail to extrude polar bodies. The question arises as to whether in such cases the first cleavage spindle develops *de novo* or whether it is directly continuous with the first polar spindle. Evidence of the similarity of the first cleavage spindle of such eggs and the normal first polar spindle has been previously presented (Tyler, 1932). In the results presented here it was shown that suppression of the polar divisions enables eggs to divide which would not ordinarily do so. The cytological work is insufficient to determine whether when the polar divisions are suppressed the first maturation spindle is converted into the first cleavage spindle. The two separate asters observed when the polar bodies are suppressed may have been derived from the poles of the first maturation spindle or may have arisen *de novo*. The fact that similar treatments applied after polar body extrusion do not produce such effects favors the former view, but in the absence of more detailed cytological evidence the question as to the origin of amphiaster in the doubly treated eggs still remains open.

The double treatment used here obviously differs from Loeb's classical double treatment for sea-urchin eggs. In these experiments the agent used for the second treatment was of the same type as that used for the first; and its effect was to enable eggs to divide by suppressing the polar bodies. Moreover, Just (1922) has clearly shown that in the sea-urchin egg only a single treatment is necessary, whereas for several different agents used for single treatments on *Urechis*, the optimally activated eggs do not divide. Thus hypertonic sea water alone gives similar results to hypotonic sea water. Hypertonic sea water was also tried on optimally activated eggs after the extrusion of the polar bodies, but no cleavage occurred.

SUMMARY

1. The polar bodies can be suppressed in artificially activated eggs of *Urechis* by means of a second treatment with dilute sea water.

2. The treatment must be applied before the time of extrusion of the first polar body and continued until after the time of extrusion of the second.

3. The eggs in which the polar bodies are thus suppressed undergo cleavage whereas ordinarily they would not do so.

4. Similar second treatments applied after the time of the extrusion of the second polar body do not induce cleavage.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

V. The Rate of Hemolysis in Hypotonic Solutions of Electrolytes

M. H. JACOBS AND ARTHUR K. PARPART

(From the Department of Physiology, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

I

In an earlier paper in this series (Jacobs, 1932) it has been shown that, on the assumption that the rate of entrance of water into the erythrocyte in accordance with simple osmotic laws is the factor of chief importance in determining the rate of osmotic hemolysis, the theoretical relation between the time at which some given degree of hemolysis is attained and the osmolar concentration of the surrounding solution ought to be given by the equation:

$$t_h = \frac{1}{k} \cdot \frac{V_0}{A}\left(\frac{c_0}{C^2}\ln\frac{c_0 - C}{c_0 - CR} - \frac{R-1}{C}\right) \tag{1}$$

or, if the external medium be water alone, by

$$t_h = \frac{1}{k}\,\frac{V_0}{2c_0A}\,(R^2 - 1), \tag{2}$$

where c_0 is the osmolar concentration of the solution in osmotic equilibrium with the normal erythrocyte, R the ratio of this concentration to that which will just cause the given degree of hemolysis, C the osmolar concentration of the external medium, V_0 the initial effective osmotic volume of the cell, A its area (assumed to be constant—a not unreasonable assumption in the case of the biconcave erythrocyte) and k the permeability constant of the erythrocyte for water; that is, a numerical measure of the amount of water that would with unit difference in osmotic pressure between the cell and its surroundings pass through unit surface in unit of time. Since V_0 and A are frequently not accurately known separately, the expression kA/V_0 may for many purposes be used as a secondary constant, k', whose calculated values over a range of concentrations give indication in the same way as do those of k of the applicability of the equations in question.

In the case of hypotonic solutions of non-electrolytes, it has already been shown (Jacobs, 1932) that the observed times of hemolysis over a wide range of concentrations are in fairly good agreement with those predicted by means of the equations, if allowance be made for a decided increase in the " osmotic resistance " of the cells produced by exposure to such solutions. Since there is some reason to believe that this increase in resistance may itself be osmotic in nature, there is no need at present to postulate non-osmotic factors to account fairly well for the observed results with non-electrolytes. In the case of electrolytes, however, which will be discussed in the present paper, conditions are somewhat different. In passing from water through a series of hypotonic solutions of, for example, NaCl of increasing concentration, the properties of the erythrocyte undergo a change, expressed quantitatively by a change in the value of the calculated permeability constant, which seems to depend on other than osmotic factors. Above a certain concentration—roughly 0.02M in the case of NaCl—the behavior of the erythrocyte is in excellent agreement with that predicted by means of the equations; that is to say, a constant calculated value of k' is obtained. Below this point, however, there is a fairly rapid increase in the value of k' with decreasing concentration which ceases only at very great dilutions of the electrolyte. This inconstancy of k', which almost certainly depends upon non-osmotic factors, and which is influenced to a striking extent by the valence of the cations present in the solution, has been very briefly mentioned in a previous preliminary paper (Jacobs, 1930) but has not hitherto been discussed at any length. We believe that it is of possible significance not only in connection with the problem of hemolysis but with certain larger ones having to do with the general question of cell permeability as well.

In the experiments here described, as well as in others omitted for lack of space, the blood used was that of the ox, obtained from freshly slaughtered animals, defibrinated immediately, and kept until needed in a refrigerator. A smaller number of experiments on the blood of man and of several other mammals gave essentially similar results. All observations were made at 20° C. ± 0.2° with the employment of exactly the same technique as that already described in the fourth paper of the present series (Jacobs, 1932), which may be consulted for further details.

II

In Fig. 1 are presented the results obtained on the same sample of blood with sucrose on the one hand and with NaCl on the other. In order that the results may be strictly comparable osmotically, the observed times of hemolysis are plotted as ordinates, not against the concentrations of the two solutions, but rather against their freezing point depressions. The latter were calculated by the empirical equation for NaCl:

$$\Delta = 3.6C - 1.3C^2$$

and for sucrose

$$\Delta = 1.86C + 0.2C^2,$$

which for the concentration range actually employed give a fairly satisfactory agreement with published freezing point data.

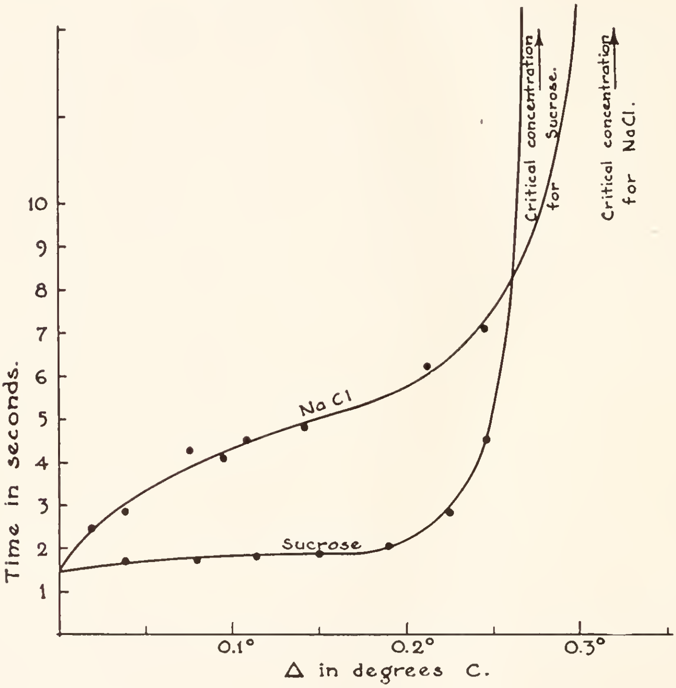

FIG. 1. Rate of hemolysis of ox erythrocytes at 20° C. in solutions of sucrose and of NaCl. One part of blood to approximately 500 parts of solution. Ordinates represent times of 75 per cent hemolysis in seconds and abscissæ calculated freezing point depressions of solutions.

Two things are immediately apparent from the figure. The first is that over most of the range covered by the experiments hemolysis occurs far more slowly in NaCl solutions than in those of sugar of the same osmotic pressure. This difference is especially striking in the most dilute solutions (e.g., of $\Delta = 0.1°$ or less) where the osmotic effect of the solute as calculated by equation 1 is almost negligible, and where the effect actually observed with non-electrolytes is equally insignificant, but where that found with electrolytes is very pronounced. As will be shown later, this electrolyte effect, which is exerted on the rate of hemolysis rather than on the position of final equilibrium of the system, is especially marked when the valence of the cations present is greater than one.

The second difference between the two curves to which reference has been made briefly above and at greater length in the preceding paper (Jacobs, 1932) is the lower critical hemolytic concentration, *i.e.,* the higher osmotic resistance of the cells, in the case of the non-electrolyte solution. In this particular case the value of Δ for which 75 per cent hemolysis just failed to occur was 0.324° for NaCl and 0.280° for sugar. This effect, which is of the "equilibrium" type, is obviously in the opposite direction from that of the first or "rate" effect, since electrolytes within the range where it is operative tend to favor rather than to oppose hemolysis. Because of the different natures of the two effects, the curves in Fig. 1 cross at a Δ value of about 0.26° for which the time of hemolysis is equal in the two solutions. Above this point there is a relatively narrow concentration range within which hemolysis actually occurs more rapidly in the presence than in the absence of the non-electrolyte. It is to be noted, therefore, that the observed rate of the hemolytic process may be affected by a mere shift in the position of final equilibrium of the system. Similar cases have been discussed elsewhere by the authors (Jacobs, 1928, 1931; Jacobs and Parpart, 1932).

The curve for NaCl in Fig. 1 shows very clearly the general relation between the concentration of a typical electrolyte solution and the time required for it to produce hemolysis; but for a more exact analysis of the extent to which such results are in agreement with osmotic laws it is necessary to employ more strictly mathematical methods. In Table I there have, therefore, been calculated by means of equations 1 and 2 for experiments with NaCl involving three separate samples of blood, values of the constant k', whose meaning is explained above and whose constancy over a given range may be taken as an indication of the applicability for this range of simple osmotic laws. The value of R employed for the calculations in each case was taken as the ratio of the freezing point depression of ox plasma (approximately 0.58° C.) to the freezing point depression of the NaCl solution in which, for the blood in question, the final degree of hemolysis was 75 per cent; this critical hemolytic concentration being determined for each sample of blood by a separate experiment. It was mentioned in the previous paper that a greater constancy of k' is obtained with non-electrolyte solutions if a somewhat smaller value of R than this be employed; but the theoretical justification for this latter procedure is rather questionable, and in the calculation in that paper of the true permeability constant, k, the same R was used as that here adopted. It should be emphasized that in view of the complexity of the material and of the various simplifying assumptions made in deriving the equations a perfect agreement between theory and observation is never to be expected. For the present, therefore, it seems advisable to use the value of R which is most simply defined and most easily determined, even though a slightly different value may fit the data rather better in some particular cases.

TABLE I

Effect of the concentration of NaCl solutions on the time required for 75 per cent hemolysis of ox blood at 20° C. One part of blood to approximately 500 parts of solution. Each time is the average of four determinations.

Concentration	Δ	Experiment 1 $R = 1.63$		Experiment 2 $R = 1.79$		Experiment 3 $R = 1.69$	
		Time *seconds*	k'	Time *seconds*	k'	Time *seconds*	k'
0.00	0.000	1.35	1.06	1.42	1.34	1.30	1.23
0.005	0.018	2.48	0.62	3.00	0.64	2.28	0.73
0.01	0.036	2.72	0.58	3.75	0.55	2.68	0.64
0.02	0.072	3.60	0.48	4.82	0.48	3.78	0.51
0.03	0.107	4.70	0.41	5.65	0.46	4.68	0.46
0.04	0.142	5.12	0.42	6.15	0.48	5.05	0.48
0.05	0.177	5.65	0.43	6.88	0.49	5.60	0.50
0.06	0.211	6.40	0.44	7.85	0.52	6.68	0.48
0.07	0.246	7.80	0.44	10.62	0.47	7.48	0.53
0.08	0.280	11.02	0.39	35.90	0.20	10.78	0.48
0.09	0.314	30.22	0.20	—	—	130.	0.06

It will be noted in Table I that the value of k' for water alone in all three experiments is relatively high, *i.e.*, 1.06, 1.34, and 1.23, respectively. These values may be compared with those of 1.16 to 1.48 found in the previous paper, when R was similarly determined, for water and for a wide range of concentrations of sugar solutions. It will be further noted that whereas with the non-electrolyte discussed in the earlier paper no appreciable change in k' occurred in passing from water to solutions of a concentration of, say, 0.04M, in the case of NaCl, an enormous change appears on passing to a concentration of only 0.005M; and a further, though much slighter, change by an additional increase in the concentration to 0.01M. Even allowing for the fact that the osmotic pressure of an NaCl solution may be twice as great as that of a sugar solution of the same concentration, it is evident that the striking retardation of hemolysis caused by very dilute solutions of NaCl can scarcely be osmotic in nature.

Passing over the narrow range of concentrations from zero to 0.01M or 0.02M, within which k' undergoes a considerable change in magnitude, we find that for all higher concentrations up to 0.07M or 0.08M the value of k' is not only remarkably constant for a given experiment but that the values obtained with different samples of blood are in good quantitative agreement. It is difficult to believe that the constancy of k' over such a wide range of concentrations is due merely to chance. The most reasonable interpretation of the facts is that within this extensive range the concentration of an NaCl solution is related to the time of hemolysis by simple osmotic laws, as has already been found to be the case (with certain limitations) with non-electrolyte solutions. It is to be noted, however, that the value of the constant for NaCl solutions is only between one-half and one-third as great as for water and for non-electrolyte solutions. The same relation holds for the true permeability constant, k, which, for a given type of blood, is always a definite multiple of k'.

As to the complete lack of agreement between the last value of k' in each series with the remainder, it may be said that determinations of rates of hemolysis in solutions lying so close to the critical hemolytic concentration are notoriously unreliable, as has been pointed out by one of the authors elsewhere (Jacobs, 1928). Successive determinations under such conditions, even when carefully made, show such relatively enormous differences as to render exact quantitative work in this region almost hopeless. It is not unlikely that the very low values of k' at the highest concentrations, where the time of hemolysis exceeds about 10 seconds, may be significant, possibly indicating an escape of salts from the cell with a consequent retardation of hemolysis (see in this connection Ponder and Saslow, 1931); but in view of the difficulty of obtaining accurate data under these conditions we prefer to leave this point unsettled for the present. The important fact remains, nevertheless, that over a wide range the effect of the concentration of NaCl solutions on the rate of hemolysis is in good agreement with that demanded by simple osmotic laws.

III

Turning now to the region of the lowest concentrations (*i.e.*, all below about 0.02M), it is apparent that in this region small changes in the concentration of the electrolyte solution affect the rate of hemolysis in a manner that is not at all in agreement with equations 1 and 2. As a matter of fact, such effects extend to much more dilute solutions than any included in Table I and are, as will now be shown, intimately related to the valence of the cations present.

In Fig. 2 are presented the results of a typical experiment with a single sample of blood in which the time of hemolysis was determined in various hypotonic solutions of NaCl, Na_2SO_4, $CaCl_2$, $MgCl_2$ and $MgSO_4$. Since over most of the range employed osmotic effects must obviously be very slight, actual concentrations rather than freezing point depressions are used in the figure as abscissæ. Furthermore, in order that a wide range of concentrations, including those of a number of extremely dilute solutions, may be covered, the concentrations are plotted logarithmically, *i.e.*, equal distances along the axis of abscissæ are taken to represent equal multiples of concentrations rather than equal arithmetical increments. The figure is therefore comparable with those of Loeb (1922) to which reference will be made below. Included in the figure for comparison is a curve, labeled *S*, which indicates the calculated, and also approximately the observed, effects of sugar solutions having the osmotic pressures of the indicated concentrations of NaCl. It will be noted that the true osmotic effects, which alone are found in such solutions, are entirely negligible over most of the range

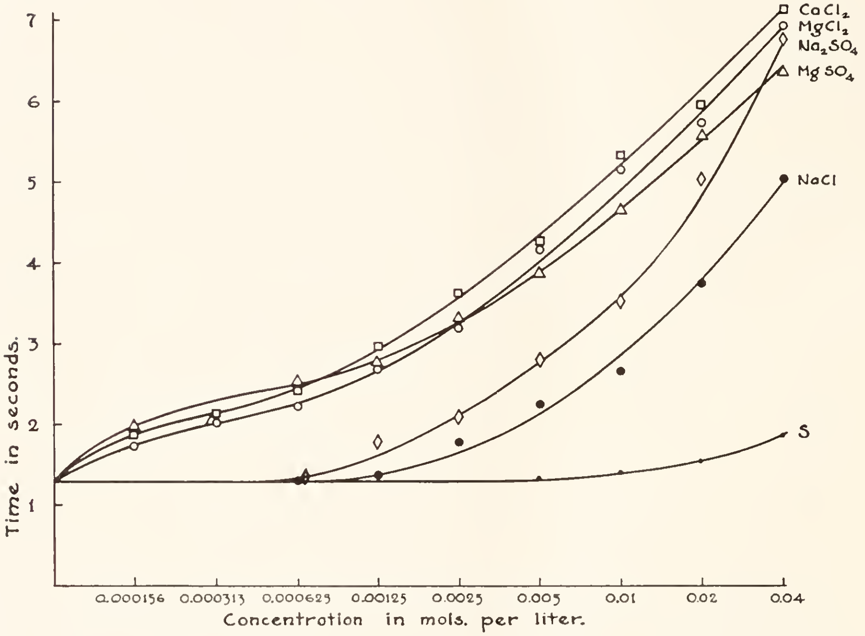

FIG. 2. Rate of hemolysis of ox erythrocytes at 20° C. in solutions of various salts. One part of blood to approximately 500 parts of solution. Ordinates represent times of 75 per cent hemolysis in seconds and abscissæ concentrations of solutions in mols per liter.

covered by the figure and that most of the effects of the electrolyte solutions must therefore be of a different nature.

An inspection of Fig. 2 brings out several additional points of interest. The first is that the salts fall into two sharply-separated groups, both with respect to the concentration at which a visible retardation of hemolysis first appears and with respect to the magnitude of the retardation at any given concentration. Thus, with $CaCl_2$, $MgCl_2$ and $MgSO_4$ a retardation of the order of 0.5 second or 40 per cent is present at a concentration of 0.00015M. A similar retardation is not reached with NaCl and Na_2SO_4 below a concentration of approximately 0.003M, and no detectable effect of any sort is found with either of the latter salts, or with KCl, which was studied in other experiments, below a concentration of about 0.001M. Throughout the entire range employed the relatively greater effectiveness of the salts of Ca and Mg is most marked. With salts of this type the valence of the cation appears to be the factor of chief importance, since there is little difference between $MgCl_2$ and $MgSO_4$.

In the case of NaCl and Na_2SO_4, both of which are rather widely separated in their properties from the salts just mentioned, it would appear that Na_2SO_4 is considerably more effective at a given concentration than is NaCl. This difference is probably to be attributed to the fact that the salt of the dibasic acid furnishes twice as many cations as that of the monobasic acid, the cation being, as already indicated, the ion of chief effectiveness in influencing the rate of hemolysis. If in plotting the two curves the concentrations of the Na ions had been used as abscissæ rather than the molecular concentrations, the curve for Na_2SO_4 would have been shifted to the right by an amount equal to that between two successive indicated concentrations; and in that case the two curves would have almost coincided. In several other experiments, not described here, the times of hemolysis for Na_2SO_4 in the region below 0.01M where osmotic effects are negligible were found to be somewhat below those for NaCl at the same $Na^{\cdot}$ ion concentration. In other words, with the same concentration of $Na^{\cdot}$, SO_4'' at times seemed, if anything, to favor hemolysis as compared with Cl', though it is to be noted that the concentrations of the two anions under these conditions were no longer the same and the differences were at best slight.

In the case of trivalent cations, a number of experiments have been made with $Al^{\cdot\cdot\cdot}$, but the results are too complex to be discussed here, since they involve $H^{\cdot}$ ion effects, agglutination of the erythrocytes, and other complications that have little bearing on the present problem. It may be mentioned, however, that in its ability to retard hemolysis at very low concentrations, $Al^{\cdot\cdot\cdot}$, under proper conditions, may very considerably exceed the bivalent ions. With it a distinct retardation of hemolysis is at times obtained at concentrations as low as 0.00001M. The rather complicated nature of the effects of Al salts upon the erythrocyte will be discussed in detail elsewhere.

In addition to the experiments here described, a considerable number of others of the same general type have been performed. Because of the great rapidity of the hemolytic process in water and very dilute solutions, the quantitative accuracy of such experiments is not always as great as might be desired, and there are some slight discrepancies from experiment to experiment; but, on the whole, the results are in very satisfactory agreement and bear out the conclusion here reached, namely, that in dilute solutions cations tend to retard osmotic hemolysis in some non-osmotic manner with an effectiveness that increases greatly with an increase of their valence from one to two, and that anions have comparatively little influence on the process, though in some cases they seem with increasing valence slightly to favor it.

IV

As to the cause of the retardation of hemolysis produced by adding to distilled water electrolytes in concentrations from 0.01M to 0.0001M or even lower, it may be said with a fair degree of certainty that the osmotic pressure of the external solution in such cases is a factor of little or no significance. This is indicated not only by the negligible osmotic effects of such solutions as calculated by means of equation 1, and as actually observed in the case of non-electrolytes, but by the enormous differences in the effectiveness of, for example, NaCl and $MgSO_4$ at the same concentration, or of NaCl and $CaCl_2$ at the same freezing point.

The possibility nevertheless suggests itself that while in such cases the external osmotic pressure is of no importance, there might conceivably be produced by the solutions some indirect osmotic effects on the cells themselves which would influence the rate of the hemolytic process. We have already pointed out (Jacobs and Parpart, 1931) that the erythrocyte is unique among cells in the readiness with which its internal osmotic pressure is affected by apparently insignificant external changes of different sorts. Unfortunately for this explanation, such effects as might conceivably be produced in this way are, in the present case, in the wrong direction. As shown by the difference in the critical hemolytic concentration for electrolytes and for non-electrolytes (see Fig. 1), the " equilibrium " effect of electrolyte solutions is in the direction of favoring rather than of opposing hemolysis. An osmotic explanation of the observed results, either direct or indirect, seems therefore definitely to be ruled out.

A more plausible explanation, because it suggests analogies in both living and in non-living systems, is that the rate of entrance of water into the erythrocyte is affected by low concentrations of ions in a manner similar to that observed by Lucké and McCutcheon (1929) in the case of the *Arbacia* egg and by Loeb (1922) in the case of collodion-gelatin membranes on the alkaline side of the isoelectric point of the gelatin. The former workers have reported that cations inhibit the passage of water into the *Arbacia* egg to an extent which increases with their valence, while anions behave in the opposite manner. In the case of collodion-gelatin membranes, where the factors concerned are obviously of a very simple physico-chemical nature, the results obtained are much the same; the nature of these effects has been discussed at length by Loeb. The erythrocyte differs from both the *Arbacia* egg and the artificial membrane in the much less prominent, and indeed somewhat doubtful, effect upon it of anions as compared with cations; but the striking difference between the ions of the alkali metals, on the one hand, and those of the alkaline earths on the other is found in all three cases, and may conceivably be due to the same causes.

An alternative explanation is that the effect of ions is on the rate of escape of hemoglobin from the cell rather than on the rate of entrance of water into it (see in this connection the discussion by Jacobs and Parpart, 1932, of the effect of narcotics on hemolysis). This explanation, however, while not completely ruled out by the existing evidence, seems to us to be less probable than the other one in view of the fact that the "equilibrium" effect of electrolytes on hemolysis, unlike that of narcotics, is in the opposite direction from the "rate" effect. Whatever the explanation of the effect of traces of electrolytes on the rate of hemolysis may ultimately prove to be, however, the observed facts are themselves entirely definite; and the non-osmotic factors shown to be concerned in the process would seem to be worthy of consideration in connection with theoretical discussions of the nature of cell permeability.

SUMMARY

1. In NaCl solutions of concentrations from about 0.02M to 0.07M or 0.08M the rate of hemolysis of ox blood is related to the concentration of the solution as if the process were governed by simple osmotic laws.

2. The permeability constant for water over this range is between one-half and one-third as great as that previously found for non-electro-

lyte solutions. At concentrations below 0.02M the calculated "constant" changes with the concentration of the solution in a manner indicative of the presence of non-osmotic factors of some sort.

3. The retarding effect upon hemolysis of dilute solutions of electrolytes increases rapidly with the valence of the cations present. The valence of the anions is much less important but, if anything, acts in the opposite sense.

4. The tentative suggestion is offered that under certain conditions ionic forces may modify to an appreciable extent the rate of the osmotic intake of water by the erythrocyte.

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HIBERNATION AND DIAPAUSE

PHYSIOLOGICAL CHANGES DURING HIBERNATION AND DIAPAUSE IN THE MUD-DAUBER WASP, SCELIPHRON CÆMENTARIUM (HYMENOPTERA)

JOSEPH HALL BODINE AND TITUS C. EVANS

ZOÖLOGICAL LABORATORY, STATE UNIVERSITY OF IOWA

It has been known for some time that many organisms enter periods of inactivity during winter or upon exposures to low temperatures. In some cases it has been clearly demonstrated that periods of rest or diapause are quite independent of external temperatures for their occurrence. The parts played by heredity and environment in these phenomena have also been much discussed. Quantitative physiological observations on single individuals during these periods of quiescence, however, have been carried out on but few forms and particularly is this true for lower forms, especially the insects (Uvarov, 1931; Dreyer, 1932; Ashbel, 1932, etc.). The present paper is concerned with results of a detailed study of certain physiological changes taking place during the developmental life cycle of the common yellow-legged mud-dauber wasp, *Sceliphron cæmentarium.*

The mud-dauber wasp, *S. cæmentarium,* is extremely favorable material for physiological investigations since it can be readily obtained in its developmental stages in large numbers and is easily kept in the laboratory with a minimum of care. Its life-cycle is relatively simple. After completion of the mud nest, spiders are captured, paralyzed, and put into the individual cells of the nest. A single egg is laid by the female wasp on the abdomen of the first spider introduced. Other paralyzed spiders are added to the cell as food for the developing larva and the cell is then sealed. The egg hatches in a very few days (depending on external temperature) and the larva eats voraciously of the enclosed spiders and quickly attains the stage at which it spins a cocoon about itself. Within this cocoon case the animal goes through the remainder of its larval, prepupal and pupal life and eventually emerges as an adult wasp. The length of the larval stage is of considerable interest, since, normally, animals hatching late in the season (August–September) hibernate in this stage. Larvæ from eggs laid early in the summer (June–July), in most instances, do not go through hibernation in the larval stage but develop uniformly, emerging out-of-doors in from 19 to 25 days (Rau, 1918). It is reasonable to assume that two varieties are normally produced—one that goes through development from egg to adult at a fairly uniform rate with no marked periods of cessation—another that normally hibernates out-of-doors in the larval stage and thus does not develop during such periods. Since it is almost impossible to collect all the eggs laid by a single female wasp during the entire laying period, one cannot say definitely that both types of eggs are laid by the same individual. Indirect evidence would seem to indicate, however, that eggs laid early in the season invariably go through development without a cessation while those laid later in the season are usually of the diapause type. A somewhat similar observation for the codling-moth has been reported by Glenn (1922), Shelford (1927), and others. Both types of individuals have been obtained in Iowa as well as from New Jersey, Pennsylvania, Maryland, and Texas. Experimental materials for this investigation were taken over a period of three successive years.

The procedure followed in obtaining animals was as follows: The mud-nests were collected at intervals throughout the year and larvæ in various stages of their developmental life cycles, as far as diapause was concerned, were thus obtained. The animals were completely removed from the cocoon cases and kept separately in shell vials or gelatin capsules at known constant temperatures throughout the experiments. Eggs laid early in the season (July) were obtained at the time of laying and after hatching were fed paralyzed spiders taken from the nests. The larvæ in these experiments were kept at constant temperatures and grew in quite a normal fashion. It was thus possible to obtain in this manner accurately timed organisms for comparison with those taken at random. Inasmuch as the last larval stage is a non-feeding one the organism is relatively easy to keep under laboratory conditions. Body weights and morphological and physiological histories were kept for individual larvæ. Oxygen consumption was determined by the modified Krogh manometer (Bodine, 1929). But one larva was used at a time and this always in the same manometer throughout the period of the experiment. Some 225 to 250 larvæ have been individually studied. Inasmuch as the results obtained are qualitatively similar it seems desirable to express them graphically. This method shows most clearly the general course of the physiological and other changes followed by the larvæ throughout their development.

EXPERIMENTAL RESULTS

I. *Non-diapause Type of Organism*

Oxygen consumption and body weight changes during the entire developmental life cycle of non-diapause individuals have been studied. All results obtained have been qualitatively similar so that only typical cases will be presented. At 28° C. the length of time required for the entire development from laying of egg to emergence of wasp is approximately 25 to 28 days. The oxygen consumption of the egg during development steadily increases up to the time of hatching. During the active feeding and growing periods of the larva, body movements are so marked that satisfactory measurement of the rates of oxygen consumption can not be obtained. After the spinning of the cocoon, the animal becomes less active and it is largely for this reason that the results presented in this paper begin at this point in the organism's development. Prior to and during the spinning of the cocoon, the alimentary canal is emptied of waste materials and this too adds much to the desirability of beginning measurements of oxygen consumption rates and changes in body weights at this stage.

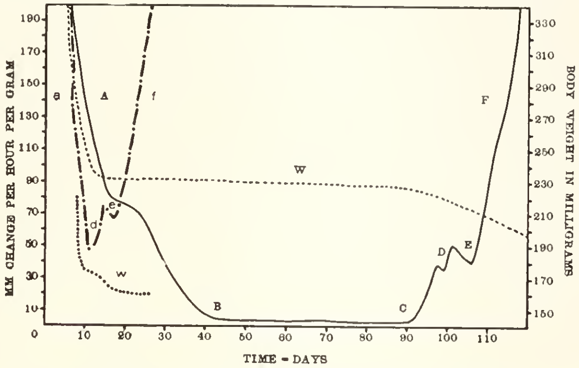

FIG. 1. Rates of oxygen consumption and body weights during the development of diapause and non-diapause types of animals at constant temperature of 28° C. Ordinates at left, millimeters change of manometer fluid per hour per gram organism (same manometer used throughout experiment; to convert readings into actual amounts of oxygen, results are multiplied by factor for the manometer in question). Ordinates at right, body weight in milligrams. Abscissæ, time in days indicated. Letters indicate different periods in life cycle of organisms. Large letters for diapause type, small ones for non-diapause type. *A* = spinning of cocoon. *B* = beginning of diapause. *C* = end of diapause. *D* = formation of prepupa. *E* = pupation. *F* = pigmented pupa ready to emerge. *W* = body weight. —— · —— = oxygen consumption, non-diapause type. ———— = oxygen consumption, diapause type. · · · · · = body weight, non-diapause type. - - - - - = body weight, diapause type. For further description, see text.

In Fig. 1 the rates of oxygen consumption and body weights of a typical non-diapause type individual are graphically shown. From an inspection of this figure it will be noted that the rates of oxygen consumption during the spinning of the cocoon are at first high but after spinning they quickly drop to a minimum. This minimum value is at the time the animal reaches the prepupal stage. After the prepupal stage an increase in the rate of oxygen consumption occurs, during which the animal prepares for pupation. During pupation a drop in rate of oxygen consumption again takes place and this is followed by a steady and marked increase up to emergence of the wasp. This drop in oxygen consumption rate during pupation, or the so-called U-shaped oxygen consumption curve, is quite characteristic for this phenomenon since it has been reported for many other forms undergoing complete metamorphosis (Taylor and Steinbach, 1931, and others). The prepupal drop in the oxygen consumption curve is equally characteristic but seems to have been reported for but few forms (Fink, 1925). It is thus seen that during the development of the wasp definite cycles or rhythms in rates of oxygen consumption occur which are closely correlated with the morphological stages through which the animal passes. Changes in body weight are also of considerable interest (Fig. 1, curve *w*). During the spinning of the cocoon a rather marked drop in weight occurs, due largely to the emptying of the alimentary canal. After this, loss in body weight is gradual but continuous up to the time of emergence.

II. *Diapause Type of Organism*

As noted above, eggs laid late in the summer (August–September) usually produce animals showing a diapause. These organisms are quite similar to the non-diapause variety in their development, the most striking difference being the length of time necessary for development at constant high temperatures (20–35° C.). In Fig. 1 there is graphically represented for a diapause individual rates of oxygen consumption and body weights during developmental stages comparable to those described above for the non-diapause organism. From an inspection of these curves it will be noted that a marked decrease in rate of oxygen consumption down to a minimum value occurs during the spinning of the cocoon and in preparation for diapause. This minimum value at constant high temperatures (20–35° C.) is always considerably lower than that reached by the non-diapause form even though the morphological stages are similar in both cases. During diapause at constant high temperatures (20–35° C.) minimum rates of oxygen consumption are practically constant and at 28° C., as shown in Fig. 1, last some 40 to 50 days. As noted further on, this minimum rate of oxygen consumption and its duration are modified to a considerable degree by different temperatures. Changes in body weights in diapause organisms are qualitatively similar to those undergone by non-diapause individuals, the only difference being a marked period of almost constant body weight during diapause.

In general, the physiological changes through which both types of organisms pass during development are, with the exception of diapause, quite similar. The rhythmic or cyclic changes correlated with larval, prepupal, and pupal changes are strikingly indicated in both. The diapause individuals, at 28° C. (as indicated in Fig. 1), require from five to six times longer for their development. Questions as to the total amounts of energy involved in the development of the two types of individuals are of considerable interest, but since it is almost impossible to secure all the eggs from the same female, many uncontrollable factors enter which tend to make such calculations hazardous. Differences in initial body weights, amounts of food stored in nests, and similar conditions make absolute comparisons, as far as the total oxygen consumed and number of days required for development, impractical for this form. Similar experiments carried out on the eggs of the silkworm by Ashbel (1932), however, show that the amounts of oxygen consumed and the number of days required for development are more or less constant for diapause and non-diapause eggs.

III. *Reactions of Diapause to Temperature*

Inasmuch as diapause seems independent of external temperatures for its occurrence it was thought desirable to determine what the effects of different temperatures would be on its duration and intensity. Rather extensive experiments have been carried out using diapause type animals taken from out-of-door environments and determining their rates of oxygen consumption throughout hibernation and growth periods. In addition, organisms in similar physiological states have been experimentally subjected to controlled temperatures and their responses studied.

1. *Diapause animals under out-of-door temperatures.*—Diapause larvæ in the same morphological stages were collected from nests during different periods of the year, from August to April, put at constant temperature (28° C.) and their rates of oxygen consumption and growth followed. Results, typical of such experiments, are graphically indicated in Fig. 2. An inspection of this figure shows that animals collected late in August have rather marked periods of low oxygen consumption rates (diapause) similar to those pointed out previously for diapause animals kept continuously at constant high temperature (Fig. 1). With the approach of low out-of-door temperatures in November the length of low oxygen consumption rates (diapause) at 28° C. gets progressively shorter. Animals put at 28° C. late in December, after

being exposed to rather long periods of low out-of-door temperatures, show no periods of low oxygen consumption rates but develop in quite a uniform and normal fashion. That this gradual shortening of the low oxygen consumption rate (diapause) is not due to the animals being of different developmental ages when collected at different periods of the year, can be easily demonstrated. Individuals taken under identical environmental conditions early in the fall and kept out-of-doors under observation for the remainder of the year always give progressively shorter and shorter periods of low oxygen consumption rates the later they are put at constant high temperature (28° C.). In other words, exposures to low out-of-door temperatures during winter in some way or other shorten the length of diapause or diapause progresses during exposures to low temperatures. No morphological or developmental changes are evident in the animals during the periods of low oxygen consumption rates (diapause).

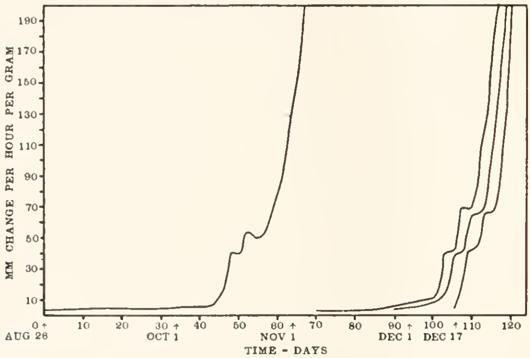

FIG. 2. Rates of oxygen consumption for diapause-type animals collected out-of-doors at different periods during·year and then placed at 28° C. until emergence. All animals in same morphological stage (hibernating larval stage) at time of collection. Ordinates, millimeters change of manometer fluid per hour per gram of organism (see note under Fig. 1). Abscissæ, time of year and days indicated. Each curve represents oxygen consumption for a single larva. For further description, see text.

2. *Diapause animals under controlled temperatures.*—Animals of known history, as far as diapause was concerned, were collected in large numbers and put at constant temperatures of 2° C. where they were left for varying periods. At different intervals of time some were taken from 2° C. and put at 28° C. and left there until emergence of the adult animal. Rates of oxygen consumption and development were carefully studied on animals thus treated. Figure 3 shows, graphically, results typical of such an experiment. From an examination of this figure it

will be noted that exposures of diapause animals to 2° C. cause a marked shortening of the period of low oxygen consumption rates (diapause) when the organism is subsequently transferred to 28° C. The degree to which this period is shortened is conditioned more or less quantitatively by the length of exposure to 2° C. Short exposures (5–10 days) cause but little change in duration while long exposures (50 + days) cause complete disappearance of the period. No morphological changes can be noted in the organisms kept for some time at 2° C. If the exposure is too long (over 3 to 4 months) a rather high mortality results. This, however, is a much longer time than necessary for complete disappearance of the period of low oxygen consumption rates (diapause). It is thus evident from such results that diapause progresses or is influenced by exposure to low temperatures (2° C.) and that such action of low temperatures out-of-doors must be a factor in the normal reaction of the organism to its environment. Temperature seems to have little influence on the occurrence of diapause but it is unquestionably a factor for its duration.

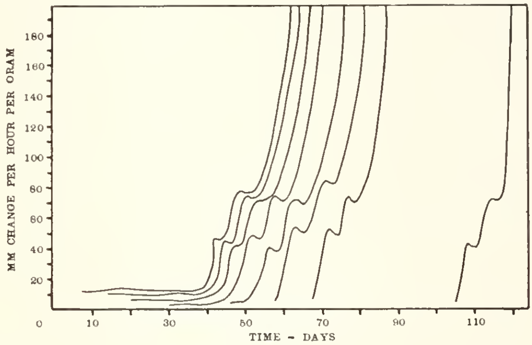

FIG. 3. Rates of oxygen consumption for diapause-type animals kept at 2° C. for varying periods and then put at 28° C. until emergence. All animals collected on same date at beginning of experiment and in same morphological stage (hibernating larval stage). Ordinates, millimeters change of manometer fluid per hour per gram of organism (see note under Fig. 1). Abscissæ, time in days indicated. All oxygen curves begin on day animal was transferred from 2° to 28° C. Period of exposure to 2° C. indicated by number of days from start of experiment to time oxygen curve begins. Each curve represents oxygen consumption for a single larva. For further description, see text.

In another series of experiments, larvæ just entering diapause were collected from out-of-doors and put at constant high temperatures (35, 28, 25° C.) as well as at 2° C. For those kept at the higher temperatures, oxygen consumption rates were determined during the entire developmental stages. In the case of those at 2° C., individuals were taken at different time intervals and transferred to 25° C., at which temperature their oxygen consumption rates and development were followed. Particular attention was given to the time necessary for diapause to disappear (as judged by the lack of low oxygen consumption rates at 25° C.) at this low temperature (2° C.) so that a relative comparison between the length of diapause at the different temperatures could be made. Figure 4 indicates graphically typical results of such a series of experiments. An examination of this figure shows that low rates of oxygen consumption (diapause) occur at the different temperatures (25–28–35° C.). Of particular note is the relative length of diapause at these temperatures. At 35° it took approximately 33 days, at 28°, 35 days and at 25°, 85 days. A temperature coefficient of approximately 2.5 has been found for the duration of diapause at these constant high temperatures. In the experiments at 2° C. it took approximately 75 to 80 days for diapause, which is about equal to the time required at 25° C. In all experiments it has been found that low temperatures (10–2° C.) are almost as efficient for the progress of diapause as are temperatures as high as 25° C. It is thus evident from these results that diapause occurs independently of the external temperature but that

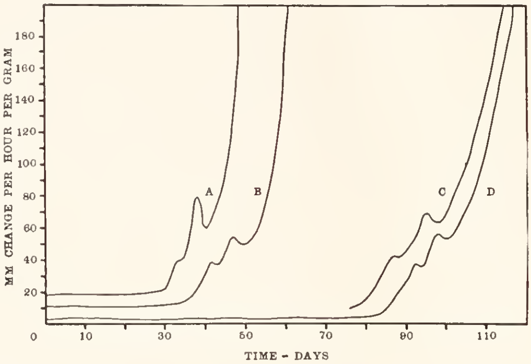

FIG. 4. Rates of oxygen consumption for diapause-type animals kept at constant temperatures as well as for those kept at 2° C. and subsequently transferred to 25° C. All animals collected at same time and in same morphological stage (hibernating larval stage). Ordinates, millimeters change of manometer fluid per hour per gram of organism (see note under Fig. 1). Abscissæ, time in days indicated. Each curve represents oxygen consumption for a single larva. Curve *A*, animals kept at constant temperature of 35° C. from beginning of experiment until emergence. Curve *B*, animals kept at 28° C. from beginning of experiment until emergence. Curve *C*, animals kept at 2° C. from beginning of experiment and then transferred and kept at 25° C. until emergence. Curve shown indicates period of exposure to 25° C. Period of exposure to 2° C. indicated by number of days from start of experiment to time oxygen curve begins. Curve *D*, animals kept at 25° C. from beginning of experiment until emergence. For further description, see text.

its duration is to a marked degree conditioned by temperature. The most unusual nature of the effect of temperature on it is the fact that low temperatures, much below the threshold values for development for this species, affect it in a marked degree.

The relative rates of oxygen consumption at different temperatures are also of considerable interest. At 28 and 35° C. the average rates expressed in terms of millimeter changes in manometer fluid per hour per gram organism are 18 and 12 respectively, while at 25° C. they are 3. At 2° C. rates are so low that it is practically impossible to get satisfactory results over long periods of time. At temperatures of 25° C. and below, rates of oxygen consumption are always extremely low in comparison with those at temperatures above this value. Such marked differences are, perhaps, due to a regulatory mechanism which enables the animal under out-of-door conditions to better meet and endure extreme fluctuations of temperature.

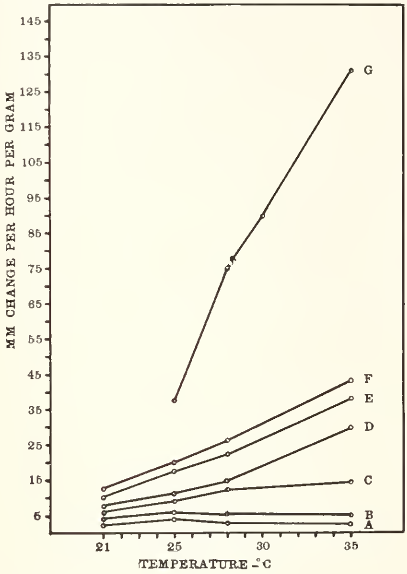

FIG. 5. Effect of temperature on rates of oxygen consumption of diapause-type animals at different periods of life cycle. Ordinates, millimeters change of manometer fluid per hour per gram of organism (see note under Fig. 1). Abscissæ, temperature, degrees centigrade. Curve *A*, animal at beginning of diapause. Curve *B*, animal in middle of diapause. Curve *C*, animal at end of diapause. Curve *D*, animal coming out of diapause entering prepupal stage. Curve *E*, animal in prepupal stage entering pupal stage. Curve *F*, animal in pupal stage. Curve *G*, animal ready to emerge. For further description, see text.

During diapause, however, responses to short exposures to different constant temperatures are quite in contrast to those for organisms in which diapause has ceased. In Fig. 5 there is graphically represented the effects of different temperatures on the oxygen consumption rates of organisms in different stages of diapause. From an inspection of this figure it can be readily seen that marked responses are given only after the period of diapause is well advanced. When reactions to different temperatures are first noted they are of small magnitudes (Fig. 5, *C–D*). As diapause progresses and begins to wane progressively greater responses are given. Curiously, temperature coefficients for the different periods are quite similar even though the actual amounts of oxygen are of greatly different magnitudes. During diapause the organism is apparently quite dependent on the particular physiological state at which it happens to be for its response to different temperatures. In early periods it is less responsive than later ones and only approaches the response normally given by non-diapause animals after the effects of diapause have disappeared.

Summary and Conclusion

1. Rates of oxygen consumption and changes in body weight during the development of diapause and non-diapause types of the mud-dauber wasp, *Sceliphron cœmentarium*, at constant temperatures are presented and compared.

2. Diapause seems, for its occurrence, independent of environmental temperatures, but its duration is conditioned to an appreciable degree by these factors.

3. Cyclic or rhythmic changes in oxygen consumption and body weight during the developmental life cycle of the wasp are pointed out.

4. Quantitative responses to temperature are modified to an extreme degree by the diapause phenomena.

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THE RESPIRATORY GAS EXCHANGE IN TERMOPSIS NEVADENSIS

S. F. COOK

(*From the Division of Physiology, University of California Medical School, Berkeley, California*)

The series of studies made in recent years by the members of the Department of Zoölogy at the University of California, as well as other investigations, have thrown much light on the relations between environmental factors and the habits of termites, particularly with respect to diet, moisture, and symbiotic microörganisms. Questions have arisen at times in connection with other ecological problems pertaining to the utilization of and the dependence on oxygen by the termite colony. In particular, it is conceivable that small differences in the oxygen tension of various habitats might influence the distribution of species and life cycle of individuals. In considering the matter from the ecological point of view, one is confronted by the difficulty that very little is known about the normal respiration of the termite itself. It was to obtain information concerning this point that the present study was made.

Several hundred specimens of *Termopsis nevadensis,* secured from the vicinity of Santa Cruz, California, were kept in open dishes, and fed a combination of moist filter paper and wood scraps. For experimental work they were sorted roughly into sizes, use being made of only the nymphs. Although such is probably not the case, there is a possibility that the respiration of the soldiers and reproductives might differ from that of the nymphs. Both oxygen consumption and carbon dioxide production were measured manometrically by the Warburg method. Usually the gas exchange of about twelve termites at a time was measured in each manometer vessel. The results were sufficiently consistent to indicate that variations between individuals were thereby eliminated. In most cases the termites were weighed and the results expressed as cubic millimeters of oxygen absorbed or carbon dioxide evolved per gram per hour. Occasionally, however, this was not necessary, particularly when the results were purely comparative in nature.

It is, of course, very difficult to keep the termites motionless and otherwise achieve basal conditions. This must be constantly borne in mind in determining respiratory quotients. Cleveland (1925) has reported a quotient of 1.0, but since he does not state the conditions under which the measurements were made, it is to be assumed that he was dealing with normal, active animals, feeding on a preponderantly carbohydrate diet, animals which would be expected to show such a quotient. However, in investigating the respiration of invertebrates, both anaërobic and aërobic, it is not necessary to achieve that particular basal state demanded in a mammal, provided the conditions are uniform throughout the entire series of experiments. With termites these conditions may be attained satisfactorily, as is shown by the following experiment.

Experiment 1.—The respiration of ten groups of termites with varying number of individuals in each was measured at 32° C. The oxygen consumption in cubic millimeters per gram per hour was: 930, 820, 850, 740, 740, 830, 760, 750, 770, 860. The extreme variation is about 20 per cent.

The possibility should not be overlooked, in dealing with large numbers of termites of somewhat different ages, that the respiratory rate may vary with age. The possibility is eliminated, however, as indicated thus:

Experiment 2.—The oxygen consumption of two groups of termites was measured at 20° C., the first group consisting entirely of individuals of an average length of 10 millimeters, the second of 15 millimeters. The consumption of Group I was 433 cu. mm. O_2/gram × hour and that of Group II was 413 cu. mm. O_2/gram × hour, the difference lying within the experimental error.

The general problem of the relation between oxygen tension and oxygen consumption has been the subject of numerous investigations. Without entering at this point into any detailed discussion of the results of this work, it may be stated that there has not yet been found any clear correlation between the two. Some animals are able to acquire and use their normal amount of oxygen at extremely low tensions; others are very closely dependent upon the tension. The characteristic reaction of each species must be determined experimentally in every case. (For reviews of the literature, reference may be made to papers by Helff, 1928, and Hyman, 1929.)

Experiment 3.—To ascertain the general relation between oxygen tension and consumption a series of comparative measurements were made with several groups of animals at 20° C., using oxygen concentrations of 100, 21, 10, 5, 2, and 0.8 per cent. For the lowest tension a tank of commercial nitrogen served with oxygen present to the extent of 0.8 per cent as an impurity. The procedure in all cases was to establish the normal rate in air and then to replace the air in the manometer vessel with the gas mixture to be investigated. Frequently two or more mixtures were introduced successively and when this was done the order was varied from group to group. Check readings were made at the end in air to insure that no injury has been done the respiratory mechanism through subjection to low or high oxygen tensions. No abnormalities were observed save in the case of 0.8 per cent. The rate in air after exposure to this tension was a little low but soon rose to the normal. The initial low rate was probably due to the immobility of the animals which is induced by very low tensions. To the immobility may also be partially ascribed the low respiratory rate *during* exposure to 0.8 per cent oxygen. Since all these rates are relative and are compared to the rate in air as normal, they may be expressed on a percentage basis, taking the rate in air as 100 per cent. The data are summarized in Table I. It will be noted that as the tension decreases, the relative oxygen consumption also decreases, but not proportionally.[1] The respi-

TABLE I

Relative Oxygen Absorption of Termopsis at Varying Oxygen Tensions

Group no.	Oxygen concentration	Oxygen absorption
	per cent	*per cent of normal*
I	100	111
II	100	93
III	100	86
IV	100	82
I	21	100
II	21	100
III	21	100
IV	21	100
V	21	100
VI	21	100
I	10	88
II	10	93
I	5	86
II	5	83
III	2	64
IV	2	73
V	0.8	26
VI	0.8	30

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[1] The effect of pure oxygen appears to be slightly inhibitory. This phenomenon has been observed in the case of other organisms but has never been satisfactorily explained. A suggestion which might be advanced is that the oxygen at high tension inhibits the respiration of the microörganisms of the gut. This fauna is killed by prolonged exposure to oxygen and may be sensitive to shorter exposures.

ration remains very near the normal until tensions below 5 per cent are reached and even with less than 1 per cent oxygen the respiration is almost one-third of its usual value. This situation indicates a very high degree of independence of the oxygen tension on the part of the termite. Furthermore, there appears to be a very well-developed capacity for utilizing extremely small quantities of oxygen. This capacity was made evident in the above experiment when the gas used was 0.8 per cent oxygen in nitrogen. For here the rate of respiration underwent a steady decrease, indicating that the oxygen tension was being materially reduced by the termites themselves and that the rate decreased along with the tension.

It seemed advisable to investigate more thoroughly this ability of the termites to utilize very small concentrations of oxygen. Therefore a series of studies was made, the data for which are consolidated in Table II. For the purpose of orientation a group of normal termites was first used with a mixture containing 0.7 per cent oxygen and the gas exchange followed for several hours (data not shown in Table II). The oxygen uptake was steady for a short time (about 30–40 minutes), then began to fall off. The decrease continued until the gas consumption ceased. But on continuing the readings it was observed that a positive pressure appeared, suggesting that now some other gas was being evolved. The rate of evolution became constant within an hour and remained so as long as measurements were continued—a matter of several hours. Since the vessel contained strong potassium hydroxide, this gas could not be carbon dioxide. The experiment was repeated with an inset of 10 per cent sulphuric acid as well as alkali, but the general course of the reaction was similar. The termites therefore evolve a gas which can be absorbed by neither acid nor alkali. No further attempt was made to determine the exact composition of this substance, but there is a strong possibility that it may be hydrogen or methane, or a mixture of both. If so, a reasonable assumption is that the microorganisms in the gut are responsible. The principal constituent of the termite diet is cellulose and the breakdown of this material is usually ascribed to the protozoa and possibly bacteria which inhabit the digestive tract. In other animals which utilize cellulose in a similar manner, such as cattle, large amounts of hydrogen and methane are produced. There is therefore considerable likelihood that we are dealing with an analogous situation in the termite, although naturally such a statement cannot be made with certainty in the absence of a quantitative analysis of the gas produced.

In order to investigate the rôle of the intestinal fauna in the production of this material—which we may call for lack of a more exact description the "undetermined" gas—a number of termites were defaunated.

Table II

Oxygen Absorption by Termites at Very Low Tensions

A. Group	I	II	III	IV	V	VI	VII
B. Previous treatment of termites	Normal	Normal	Normal	Defaunated	Defaunated	Defaunated	Defaunated
C. Initial O_2, per cent	0.7	0.7	0.7	0.7	0.7	2.08	2.08
D. Weight in mg	513	1067	457	552	1126	386	699
E. Duration of experiment in hours	6	$6\frac{5}{6}$	$7\frac{3}{4}$	$6\frac{1}{2}$	6	$10\frac{1}{2}$	$6\frac{1}{2}$
F. Net gas exchange in cu. mm.	−0.5	+52	−4.5	−83	−88	−333	−271
G. Rate of production of undetermined gas in cu. mm. per hour	11.3	19	9	0	0	0	0
H. Total production of undetermined gas in cu. mm.	79	130	77.5	0	0	0	0
I. Rate of production of undetermined gas in cu. mm. per gram termite per hour	22.1	17.8	19.7	0	0	0	0
J. Total gas in manometer vessel in cu. mm.	13,150	12,650	13,200	13,110	12,450	15,250	12,850
K. Oxygen at beginning of experiment in vessel in cu. mm. J × C	92	88	92	92	87	318	270
L. Total oxygen absorbed in cu. mm. F − H	79.5	78	74.5	83	88	333	271

This was done by the method of Andrew (1930) which consists of the application of oxygen at several atmospheres pressure. When defaunated termites are placed in 0.7 per cent oxygen there is no indication whatever that any gas is evolved. The oxygen consumption sinks to zero and remains there indefinitely. It seems legitimate therefore to ascribe the inert gas production to the intestinal fauna.

The complicating factor of formation of the undetermined gas may thus be eliminated by defaunation, but the question is introduced whether defaunated and normal termites react in the same way to low oxygen tensions. To investigate this matter and simultaneously to secure data concerning the original problem of utilization of small amounts of oxygen the following experiment was performed.

Experiment 4.—Seven groups of termites were placed in low concentrations of oxygen. Groups I, II, and III were normal, the remainder were defaunated. The first three groups and also Groups IV and V were placed in 0.7 per cent oxygen and Groups VI and VII in 2.08 per cent oxygen. The oxygen content of these mixtures was checked carefully by analysis with the Haldane gas apparatus. Then the gas exchange in each group was measured until, in the case of the first three groups, the rate of production of the undetermined gas was clearly established, and in the case of the others the oxygen consumption had ceased for at least two hours. At the end of this time the readings were discontinued and the *net gas exchange* of each group calculated. This, of course, is obtained from the initial and final manometer readings (see line F in Table II). With the defaunated groups the net exchange is equal to the total oxygen absorption, since there is no other gas concerned. With the normals the net exchange is equal to the oxygen absorbed plus the undetermined gas evolved. To find the total oxygen absorption we must subtract from the net exchange the total undetermined gas evolution. This involves the fairly reasonable assumption that the production of undetermined gas has the same constant value while oxygen is being taken up that it is observed to have after oxygen uptake ceases. This assumption, though reasonable, still remains an assumption, for definite evidence cannot be secured until a method is devised for differentially absorbing the undetermined gas in the vessel as fast as it is formed. There appears to be no method at present for doing this. If we, then, subtract the total undetermined gas evolved from the net gas exchange, we get the probable total oxygen consumption of the normal groups (see line L in Table II). Finally, since the volume of the vessels is known and also the percentage composition of the gas initially introduced, the actual initial quantity of oxygen can be calculated (see line K in Table II). The total oxygen usage may then be compared with the total oxygen available.

From the data presented in Table II the following conclusions may be drawn. In the defaunated groups (IV–VII inclusive) there is a fairly close correspondence between the oxygen absorbed and the amount initially present in the closed system. At least it may be stated that there is no significant quantity of oxygen remaining in the system when the oxygen consumption of the termites ceases. This appears to be true irrespective of the initial concentration (compare Groups IV and V with VI and VII). In the normal termites there seems to be a slight residue of oxygen, a concentration of the order of 0.1 per cent or a tension of less than one millimeter of mercury. But this difference between the normal and defaunated animals is too slight to be of significance, particularly since (1) the difference is of the order of the experimental error (as indicated by the deviations in Groups IV–VII) and (2) the assumption of a constant rate of inert gas evolution, irrespective of oxygen tension, may not be precisely consistent with the facts. In general, however, it is possible to state that both normal and defaunated termites are able to utilize substantially all the oxygen in the immediate environment even though the latter reaches exceedingly low tensions.

Under anaërobic conditions at least the production of undetermined gas is very constant (see line I in Table II) at a rate of about twenty cubic millimeters per gram termite per hour. A further study of this gas production would be of interest with respect to the composition of the gas and also its possible bearing on the problem of cellulose digestion.

The results obtained with low oxygen tensions suggest further questions: (1) What is the aërobic carbon dioxide production in normal and defaunated animals? This involves also the determination of the respiratory quotient of both types of animal. (2) Is carbon dioxide produced anaërobically, and if so, can the termites incur an oxygen debt? (Such has been found to be the case with the cockroach by Davis and Slater, 1926, 1928.)

Experiment 6.—Eight groups of termites were investigated (see Table III). Groups I–IV were normal animals. Groups V–VII were defaunated one day previously and Group VIII was defaunated two weeks previously. In every case the gas exchange was determined in air in the presence of 5 per cent KOH (line C, Table III) and then in air without alkali (line D, Table III). When no gases other than oxygen and carbon dioxide are involved the result of the first determination represents the oxygen consumption, since the carbon dioxide is quantitatively absorbed by the KOH. The observed gas exchange in the second determination then represents the difference between the oxygen consumption and carbon dioxide production. Since the former is known, the latter may be calculated (line E, Table III). It was ascertained in the previous experiment (Experiment 5) that there is no production of the undetermined gas by defaunated termites and therefore in Groups V–VIII the observed exchange of carbon dioxide and oxygen may be taken as the corrected, or true, exchange (lines H and I, Table III). With normal termites it may be assumed, as previously, that

TABLE III

Carbon dioxide production and respiratory quotient of termites. Gas exchange in all cases expressed as cubic millimeters per gram termites per hour.

A. Group and condition	I Normal	II Normal	III Normal	IV Normal	V Defaunated 1 day	VI Defaunated 1 day	VII Defaunated 1 day	VIII Defaunated 2 wks.
B. Weight in mg.	731	633	681	866	852	648	977	694
C. Observed oxygen consumption in air (KOH)	186	218	173	239	190	239	154	200
D. Observed gas exchange in air (no KOH)	11	14	21.5	22.5	−3.5	2.0	4.0	−36
E. Observed carbon dioxide production (C–D)	192	232	194.5	261.5	186.5	241	158	164
F. Observed R.Q.	1.06	1.065	1.12	1.095	0.98	1.005	1.025	0.82
G. Production of undetermined gas (average from Table II)	20	20	20	20	0	0	0	0
H. Corrected oxygen consumption in air (KOH) C–G	206	238	193	259	190	239	154	200
I. Corrected carbon dioxide production in air (no KOH) C–D	197	232	194.5	261.5	186.5	241	158	164
J. Corrected R.Q.	0.955	0.975	1.005	1.01	0.98	1.005	1.025	0.82
K. Total gas production in nitrogen	46.5	52	48.5	48	50.5	55.5	43	43.5
L. Carbon dioxide production in nitrogen (K–G)	26.5	32	28.5	28	50.5	55.5	43	43.5

under aërobic conditions there is a constant production of about twenty cubic millimeters of the undetermined gas per gram termite per hour; otherwise the respiratory quotient of the normal animals appears to be considerably over unity (line F, Table III). This factor may be allowed for and corrected values of the oxygen and carbon dioxide exchange obtained (lines H and I in Table III). Applying this correction, the values of the respiratory quotient approach very closely to unity (line J, Table III). The freshly defaunated termites also show the same approximate value, and with them no assumption concerning the production of the undetermined gas is necessary. These results confirm the statement of Cleveland that the usual R.Q. of termites is practically 1.0. It is of interest to note, however, that termites which have been defaunated for some time and consequently probably are undergoing starvation show a much lower R.Q.—in the one case here investigated 0.82.[2] This is to be expected from what we know in general of the effect of starvation on the R.Q. of other animals.

To determine the anaërobic carbon dioxide production the same termites were subjected to as low an oxygen tension as could readily be obtained, 0.7 per cent. Since it was ascertained in Experiment 5 that the consumption of this oxygen ceases or is too small to have material effect in about three hours, the termites were allowed to remain for this period in the closed vessels and then the gas exchange was measured for one hour. The readings showed a low but distinct positive variation, indicating the production of gas (line K, Table III). With normal animals part of the output (20 cu. mm./gm. × hr.) was due to production of the undetermined gas and therefore this amount was subtracted from the observed gas production to give the carbon dioxide value (line L, Table III). This value is considerably below that obtained under aërobic conditions but is evidence that some metabolic changes are still proceeding in the animal's tissues.

To summarize this experiment and answer question 1, it may be said that both normal and freshly defaunated termites produce carbon dioxide under aërobic and anaërobic conditions; that the R.Q. of both types of animal is very close to unity; and that the anaërobic carbon dioxide production while present is much smaller in both types than the aërobic production.

Experiment 7.—Three groups containing equal numbers of termites at 20° C. were placed in air and their respiration measured with and without the presence of KOH. Group I was then exposed to 0.7 per cent oxygen in nitrogen in the presence of KOH for an hour and a half, or until the oxygen was nearly exhausted, at which time air was reintroduced. The oxygen consumption began again and after a brief interval at a lower rate proceeded indefinitely at the same rate as at the beginning.

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[2] All the termites were normally mobile and active, even those which had been starved. All were therefore at the same "basal" level.

The absence of any increase in the rate above the normal precludes the possibility of oxygen debt. Groups II and III were similarly treated except that KOH was not present. Air was readmitted at the end of three hours of anaërobiosis. The respiration rates (both carbon dioxide and oxygen) returned very quickly to their normal values and remained there for several hours, at the end of which time the experiment was discontinued.

Experiment 8.—A repetition of Experiment 7 in which the results of the latter were confirmed.

It seems clear that the termite (*Termopsis* at any rate) possesses a mechanism for the continued production of carbon dioxide even in the complete absence of oxygen. That this mechanism is not identical with that which presumably obtains in mammalian muscle and elsewhere is demonstrated by the complete lack of any indication, from gas measurements at least, of oxygen debt. That the anaërobic system is rather inefficient is shown by the fact that the carbon dioxide production under such conditions is less than one-half the normal value and furthermore by the fact that the termites pass into a state of complete immobility even though they continue to respire. This state resembles acute anoxemia in mammals in that it appears very soon after a sudden exclusion of oxygen and disappears very quickly after readmission of oxygen, a matter of minutes in both cases.

TABLE IV

The Effect of Prolonged Exposure to Nitrogen

Termite group	Normal oxygen consumption	Hours exposure to nitrogen	Oxygen consumption after exposure	Condition after recovery
	per cent		*per cent of normal*	
I	100	1	89	Immediate recovery from immobility
II	100	3	99	Same
III	100	6	86	Recovery in 15 minutes
IV	100	9	95	Recovery in 20 to 30 minutes
V	100	24	99	Recovery in 1 hour
VI	100	48	96	Recovery in 12 hours
VII	100	96	42	Never recovered
VIII	100	144	16	Never recovered

It is worth while to determine how long *Termopsis* can endure complete anoxemia and still retain its capacity for normal aërobic respiration and normal activity.

Experiment 9.—Eight groups of termites, after their normal oxygen consumption was measured, were placed in a desiccator. The latter was filled with nitrogen from which the oxygen had been removed by treatment with strongly alkaline pyrogallol. At intervals the termites were removed and their respiration measured as well as their general behavior observed. The respiratory rate was measured after the termites had recovered their mobility, except in Groups VII and VIII which never recovered. The lack of mobility may therefore in part account for the low rate of oxygen consumption noted in these two groups. The effect of prolonged exposure to pure nitrogen is summarized in Table IV.

It will be observed that the first noteworthy reduction in respiration occurs in the group which had been without oxygen for four days and that this group never recovered their mobility. These termites therefore are not truly anaërobic in the same sense as, for example, yeast, which is able to survive and grow indefinitely in the absence of oxygen. Nevertheless the survival without apparent harm for two days is in itself a striking and significant phenomenon.

In addition to the ability to withstand oxygen lack, *Termopsis* shows high tolerance to the presence of carbon dioxide. The respiration of four groups of termites was measured in 20 per cent oxygen plus 5, 10, 20, and 40 per cent carbon dioxide respectively. The net gas exchange of those in the 5, 10, and 20 per cent carbon dioxide, with no alkali present in the vessels, was approximately the same as that of termites in air and the appearance of the termites otherwise was perfectly normal. It is unlikely therefore that any considerable upset occurred in their respiration. The same considerations concerning respiration apply to the group in 40 per cent carbon dioxide, but these termites soon became immobile.

The general conclusion may be drawn from all these experiments that *Termopsis nevadensis* possesses powers for meeting adverse environmental conditions far in excess of its probable needs. The natural habitat is relatively well aërated wood in which the gas tensions are probably not far from atmospheric. Nevertheless, these animals are capable of extracting practically the last traces of oxygen in a closed space and then of persisting several hours, if not days, in the absence of oxygen. Furthermore, they can endure quantities of carbon dioxide which would seldom if ever be present naturally in their environment. These conditions might occur in wet soil or in other habitats frequented by some of the other species and genera of termites. If this is so, it is possible that ability to withstand such conditions is a general characteristic of all the members of the group which persists in certain species even though the actual need is seldom encountered. A comparative study of the different species of termites with respect to their respiratory power and their environment is very desirable.

SUMMARY

The oxygen consumption of *Termopsis nevadensis* does not decrease materially with falling oxygen tension until a concentration of approximately two per cent is reached. Below this tension the affinity of the animals for oxygen is so marked that substantially all the available gas is consumed.

In the absence of oxygen the organism is able to respire anaërobically, although at a reduced rate, for as long as two days without injury. During this time the animals are in a state of immobility from which they recover soon after readmission of air. After exposure to anaërobic conditions no indication of oxygen debt was found.

These termites are able to exist and respire normally in carbon dioxide as high as 20 per cent. Higher concentrations tend to induce a condition of anæsthesia, which, however, is reversible.

Under anaërobic conditions, and possibly also in the presence of oxygen, the termites evolve an undetermined gas which may be hydrogen or methane. The production of this gas depends on the integrity of the intestinal fauna, since it is not evolved by defaunated termites.

The respiratory quotient of both normal and freshly defaunated termites is approximately unity, but in starved defaunated termites it falls considerably.

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FURTHER STUDIES OF THE AGGREGATING BEHAVIOR OF AMEIURUS MELAS[1]

EDITH S. BOWEN

Whitman Laboratory of Experimental Zoölogy, the University of Chicago

The investigations here reported are continuations of work on the rôle of the sense organs in aggregations of young catfishes (Bowen, 1931), and were directed toward securing further evidence concerning (*a*) the analysis of the factor concerned in the touch reaction; (*b*) the retention of touch and visual aggregating reflexes during prolonged isolation; and (*c*) the influence of aggregation and of isolation upon the physiological processes indicated by the rate of oxygen consumption. Taken as a whole, these studies together with those previously reported contribute to our understanding of the mechanisms concerned in the schooling behavior of *Ameiurus melas* in particular and to some extent to that of fishes in general.

Sense Organs Involved in the Touch Reaction

The reactions of aggregating young catfishes of the species *Ameiurus melas* have already been studied to determine which sense organs are responsible for receiving the stimuli that result in the formation of the aggregations (Bowen, 1931). Vision was found to be essential for the reactions concerned since blinded fishes did not aggregate and normal fishes failed to aggregate in the dark. There were indications, however, that responses to touch are also of great importance, and that the resulting stimulus may perhaps be the fundamental cause of the aggregations and that vision serves only as a means by which the fishes find one another. Individuals of this species are strongly thigmotactic; they always rest with as many points of the body as possible in contact, and in the aggregations they push against each other continually when disturbed. The components of this contact stimulus were not determined, but the early work indicated that it might contain both tactile and gustatory elements. Morphologically this is possible since (Herrick, 1902) the skin of catfishes over the whole body contains not only end organs which are sensitive to pressure but also terminal buds, corresponding to taste buds in the mouth. These are most abundant on the barblets and diminish in number toward the tail. They are sensitive to chemical stimuli through contact as contrasted with the olfactory organs, which are distance receptors (Parker, 1910).

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Observations of the behavior of catfishes in the tanks was also suggestive of a gustatory response. One catfish would often approach another, drag its barblets over it and push against it several times. This occurred to some extent when the second catfish was entirely inactive or when an active individual came in contact with a recently pithed fish. The same positive reaction was also given to goldfishes and mudminnows which were in the same aquarium with the catfishes. The response was negative to a weighted model, made of paraffin mixed with India ink to simulate a small catfish in color and form. The approach was the same as to another catfish, but the barblets were drawn over the model only once and then the fish turned away and swam off.

It was considered desirable to analyze further the contact stimulus which one catfish receives from another, and to determine the relative importance of pressure and of chemical stimulation.

An experiment was designed to determine whether there is a difference in the reactions of a catfish to a scoured stone which presumably gives no chemical stimulus and to another catfish rendered incapable of movement and reciprocal pressure. Fishes were pithed and the wound was covered with melted paraffin to prevent the diffusion of body fluids and a resulting gustatory stimulation. Each of these freshly killed fishes was placed at the side of a crystallizing dish 24 cm. in diameter, and held down by a small pebble since the lifeless bodies floated to the top if unattached. A small stone about the size of a catfish was placed at the opposite side of each dish after which a normal individual about 3 to 5 cm. long was added. Observations of the positions of the catfishes were made at 5-minute intervals, or in a few cases after a longer period; after each observation the normal fish was disturbed so that the position in the next observation was determined anew. After five series of records had been made the pithed fishes and the stones were interchanged so that a positive reaction to one place due to light or some other factor would not mask the results. In most cases the active fishes had come to rest at the end of 5 minutes, although in a few instances one or more were still active. If a fish was moving within a small area the location was noted; otherwise these cases were not included in the results.

The results are given in Table I(*a*). It is readily seen that the number of positive reactions to the pithed fish and to the stone are about the same, 24 and 29 respectively, whereas the total number of indifferent reactions where the fish was resting apart from either one totals 94. This indicates that there is no difference between the reactions to a stone and to a catfish if the latter is rendered inactive. Immediately upon the conclusion of the first series a control was run by removing the pithed fishes from each dish and substituting a normal fish. These results are shown in Table I(*b*). In 62 per cent of the cases the fishes were resting together as contrasted with 16.3 per cent in Table I(*a*). Since in these cases a response was recorded for each fish, this is equivalent to 31 per cent positive contacts per fish. The increase over the incidence (16.3 per cent) of positive contacts for fishes isolated with one pithed fish is due to stimulation of a visual nature from the moving partner as well as to reciprocal pushing after the two have met. Even with two stones present in the dish with two fishes (Table I, *c*), the fishes aggregated about as much as in the preceding arrangement where the single stone allowed less possibility of purely indiscriminate contact. In about half of these observations the two fishes were found resting together, an equivalent of 24.5 per cent positive contacts per fish, and a decided increase over the 16.3 per cent in (*a*) where an inert partner was present.

TABLE I

Reactions of catfishes to stones and to other catfishes as shown by resting positions

Condition of experiment	No. of fishes	Positive reactions		Indifferent	Total
		Fish	Stone		
a. Normal fish with stone and pithed fish	5	10	10	27	47
	5	4	6	40	50
	5	10	13	27	50
Total	15	24	29	94	147
b. Two normal fishes with stone	10	62	18	20	100
c. Two normal fishes with two stones	20	98	48	54	200

Since it had been found that catfishes responded negatively to a black paraffin model, it was possible that the small amount of paraffin upon the pithed fish might be preventing a positive reaction to the pithed fish itself. Accordingly the responses to a paraffined and to a non-paraffined stone were compared. An equal number of positive reactions, 24, was given to each object with 88 indifferent responses, so that it may be concluded that there is no definite reaction to the paraffin itself.

From the above results there is no evidence for a gustatory element in the stimulus which one catfish receives from another. When movement and pressure are eliminated the positive response is given in as many cases to a stone as to a catfish. The gustatory stimulus enters only to produce a negative reaction when some unfavorable chemical is present. Observations upon normal fishes in a dish with untreated paraffin models and similar models mixed with India ink to add color showed that a decidedly negative reaction was given only to the India ink models. To untreated paraffin models the reactions were the same as to an inactive fish. It was impossible to eliminate the sight reflex by using blinded fishes in these experiments, because a blinded fish remains active for long periods without coming to rest near any object; such fishes rest only after long activity and in the absence of recent stimulation.

Effect of Isolation upon Aggregating Behavior

The question as to whether the reactions concerned in catfish aggregations are entirely instinctive and automatic or are in part due to conditioning or at least susceptible to modification by conditioning is of interest in this simple type of social behavior found low in the vertebrate scale. Parr (1927) explained the schooling of pelagic fishes by an assumption of a simple automatic eye reflex which acts in the case of milling to produce a type of behavior with no apparent purpose. Learning among fishes has, however, repeatedly been demonstrated. Thus individuals have been trained to associate a stimulus such as light, color (Hineline, 1927), or direction (Churchill, 1916), with food, or in Goldsmith's work (1914) with the nest; and Triplett, 1901, by separating a perch from minnows by a glass partition, conditioned the perch so that after the removal of the partition it made no attempt to reach the minnows by passing the line where the partition had been. Recently Bull (1929) has shown that *Blennius pholis* can be trained to use the senses of taste and smell in the capture of food, although normally they play no part in this behavior. He has demonstrated that purposive movement in fishes can be explained by a combination of unconditioned and conditioned responses in nature as well as in the laboratory.

Two types of reactions of catfishes to one another had previously been observed (Bowen, 1931). Normally vision is the means by which individuals are enabled to come in contact with one another. In addition blinded fishes, previously grouped, were shown to respond to each other when the skin was intact by turning out of their course toward a passing fish in approximately 50 per cent of the cases where the two fishes came within two inches of each other. This reaction is apparently due to vibrations set up by the tail of the passing fish and received by the skin, and which act possibly more or less as a thigmotactic stimulus. This positive response may result only after fishes are blinded, but it serves to bring the fishes momentarily nearer one another, and may therefore be considered as a social response.

If the satisfaction of a thigmotactic response is the fundamental basis of aggregating behavior in catfishes, then it seems possible that both the above types of response might be modified if, through isolation, thigmotactic responses from other fishes were prevented for a time. The first indication that such might be the case was found in work with gradient tanks (Bowen, 1931) in which blinded catfishes, isolated from the group for a week, showed a tendency to stay in the opposite end of the tank from the group of fishes when the latter were separated from the single individual by a wide-meshed wire partition. The observed difference in behavior was statistically significant; at the same time blinded fishes which had been members of a group tended to stay in the end toward the group, although the difference from the controls was not significant with blinded fishes.

To determine whether the response of blinded fishes could be conditioned by isolation, individuals were placed in two-quart jars containing plants and fed regularly for a period of 20 days. They were then placed in groups of five in a porcelain tub and the reactions recorded for 15 minutes. At the conclusion of these observations they were kept grouped for one or more days and tested again as a control to determine whether they gave the normal number (50 per cent) of positive reactions after grouping. In almost all cases a positive reaction was given when two fishes actually touched. The few exceptions occurred when they met with some force and darted back in a negative response. In cases where the fishes passed within two inches of each other without touching, however, a difference between previously isolated and grouped fishes was evident. While the latter gave a total of 27 positive responses, 1 negative and 26 indifferent, showing 50 per cent positive reactions, isolated individuals gave 14 positive, no negative, and 38 indifferent responses with only 27 per cent positive. The difference is, however, actually even greater between the two groups; during the first 9 minutes after grouping of previously isolated fishes there was only one positive response and 30 indifferent ones. During this period there were several positive reactions to contact, which apparently served to overcome the effects of isolation, so that the positive reaction was reëstablished at the end of that time. In fact the positive responses for the next 6 minutes rose to 62 per cent of the total.

From this series it seems evident that the positive responses of blinded fishes to one another are eliminated by isolation, but are reestablished again in a few minutes when the fishes are in the same container and contact may occur. The ease with which the response of blinded fishes to vibrations caused by another fish is broken down and the fact that several minutes are required for its reëstablishment make it seem possible that this is a true conditioned response developed after the fishes are blinded, and in connection with the thigmotactic stimulus. It does not act with enough efficiency to enable the formation of aggregations, as one fish can determine the direction of another fish for only one or two turns and then loses it. In normal individuals vision directs the movements of the fishes toward one another at a greater distance than this stimulus can be felt so that it probably acts very slightly if at all in aggregations of normal fishes.

The effect of isolation upon the aggregating behavior of normal fishes was also tested by a similar series of experiments. Several attempts were made to isolate very young catfishes to determine how readily the normal response might be altered before the characteristic social reactions had become established by long use. But it was impossible to keep the very young fishes alive in the laboratory. They died in large numbers under the best conditions and none survived a spell of hot weather. Therefore most of these results were obtained upon older fishes.

The method employed in recording the reactions of the fishes to one another was similar to that for the blinded fishes except that a distance of about a foot and a half was considered the maximum within which a positive reaction was possible, since normal fishes nearly always react to one another within this distance. In all cases the two or three fishes placed in the experimental tub at one time were aggregating closely in the course of a few minutes, so that reactions were recorded only until this occurred. These were of two types, touch reactions where two fishes came in contact without any observable turning from their course when within visual distance, and sight responses where one of the fishes turned definitely toward the other. When either resulted in typical aggregating behavior it was considered positive; if not, indifferent. Usually a touch stimulus resulted in a positive response but in a few cases, almost always with previously isolated fishes, the first touch response was indifferent, but was followed shortly by a positive response often quite clearly as a result of a sight stimulus.

The results of all the experiments are given in Table II. The effect of isolation upon reactions responsible for aggregations seems to be shown most clearly by the number of indifferent reactions as compared with the positive sight reactions before aggregation is established. In each case where there were indications of a breakdown of the aggregating behavior, the fishes were kept together for a day and the experiment repeated as a control. Such procedure was necessary for comparison since normal fishes which have been isolated for a long period of time require several days before they become as active or responsive as those from a group, a condition which might affect the proportion of positive reactions.

The isolation of very young fishes for five days (Experiment 1, Table II) gave an indication of a breakdown of the aggregating responses. In Experiments 2 and 3, where young fishes about six months of age were isolated for between one and two months, the behavior was not modified and was entirely typical of fishes from the group. In Experiments 4 and 5, however, where the individuals were kept isolated for over five months, there was decided evidence for an isolation effect. Under these conditions the fishes isolated for five months gave in all 9 indifferent sight responses and 5 indifferent touch responses as compared with one of each after they had been together for 24 hours. Only 2 positive sight responses were noted before contact occurred as contrasted with 6 in the control. As the sight stimulus still acted in two

Table II

Summary of reactions of catfishes to one another after isolation

Exp.	Number of fish	Number tested together	Length	Days isolated	Indifferent reactions before positive response			Positive sight reactions before contact
					Sight	Contact	Total	
1.	6	2	1 in.	5	6	1	7	1
1(*a*).*	6	2	1 in.	0	3	0	3	0
2.	8	2	3–5 in.	38	0	1	1	3
3.	8	2	1.5 in.	52	0	0	0	6
4.	3	3	2 in.	161	4	0	4	0
4(*a*).* (from Exp. 3)	3	3	2 in.	0	0	1	1	1
5.	12	3	2 in.	161	5	5	10	2
5(*a*).*	12	3	2 in.	0	1	1	2	5

* In (*a*) of each experiment are the results when the fish were retested after one or more days together.

cases without a touch stimulus, the former was not completely eliminated, but there seemed to be a decided tendency in that direction.

Whether this sight reflex is instinctive or whether it is established soon after hatching as a conditioned response depending upon the positive thigmotactic reaction is still a question. If instinctive, however, it is apparently subject to change by the conditioning processes involved in isolation if these act for a long enough period of time. Probably also this effect can be produced more readily the younger the fishes. Unfortunately observations on the initial reactions of newly hatched fishes could not be made, hence the obvious and crucial test for the relative amounts of instinctive and conditioned elements in this behavior complex is lacking. However, the evidence indicates that conditioning plays a part in this aggregating behavior.

From a survey of the results obtained to date in the investigation of the reactions responsible for the aggregations of young catfishes, we may conclude that the two senses concerned are sight and contact with taste playing no part in spite of the acuteness of this sense in these animals. Sight is the sense by means of which the fishes normally aggregate and the visual response of catfishes may be an instinctive reaction to one another. Touch, however, probably has the more basic rôle, since the sight response can be diminished somewhat in effectiveness by isolation and is reëstablished by contact. The mutual pressure of the fishes due to pushing seems to give the stimulus which maintains these aggregations.

Respiratory Behavior of Grouped and Isolated Catfishes

It has been shown that grouping has an effect upon the respiratory rate of animals. Thus Allee (1926 and 1927) has found that in land isopods and the brittle starfish, *Ophioderma brevispina,* at least when out of the breeding season, the rate of respiration, as measured by the amount of oxygen consumed, is lower per animal for grouped individuals than for isolated ones for the first few hours. The rate of respiration decreases more rapidly for the isolated animals, however, so that after several hours these are respiring more slowly than the grouped ones. There is evidence that, in the case of the starfishes at least, the opportunity for physical contact afforded by the other individuals present is responsible for the group effects upon respiration and for the longer survival of the individuals composing it. Schuett (1931) has investigated this phase of respiratory behavior among goldfishes, guppies, mudminnows and *Fundulus heteroclitus,* and has obtained results similar to those of Allee for land isopods and brittle starfishes. In his shorter experiments, which covered usually one to five hours, the group consumed less oxygen per fish than did the isolated individuals. Working independently, I have been able to confirm Schuett's results with goldfishes, the only one of his species tested.

It seemed desirable to determine whether any difference exists between the respiratory rate of isolated and grouped individuals of aggregating young catfishes, especially in view of the part that contact plays in such reactions. Accordingly experiments were run with both normal catfishes and with catfishes which had been blinded several days previously in order to eliminate the important effect of vision upon aggregating behavior.

The method followed the technique of Schuett. Each experiment was performed in parallel with normal and blinded fishes. In each case, four single fishes were placed in individual Erlenmeyer flasks, holding one liter, and four were placed together in a similar flask. The flasks were then arranged parallel to two windows to equalize the normal effect of light upon the behavior of catfishes. The flasks had previously been filled with well water which in most cases had been allowed to stand overnight to come approximately to room temperature and to saturation with air, although in the first experiments air was bubbled through the water. In all of the experiments the oxygen tension of the initial samples varied only between 4.5 and 6.5 cc. per liter of water; in this range the rate of oxygen consumption is independent of the oxygen tension (Schuett and citations). After the introduction of the fish, a layer of heavy mineral oil was poured into the neck of the flasks to prevent gaseous exchange at the surface. By means of glass siphoning tubes kept in the flasks, samples of about 15 cc., known to within 0.05 cc., were withdrawn for analysis for oxygen by the method of Winkler. One sample was taken immediately after the introduction of the animal and a second one one hour later. A control flask without fish was sampled similarly. The external end of the siphon terminated in a piece of rubber tubing closed by a clamp. In the first half of the experiments the siphons reached to the middle of the flasks. In the last ten experiments longer siphons were employed and the water was stirred with the siphon one minute before sampling. No difference was noted in the results with the later modification of the technique. The fishes to be used in these experiments were kept on the experimental table in glass-walled aquaria to avoid excessive stimulation while being transferred to the flasks.

Table III shows the results of the experiments. The total oxygen consumed by four isolated fishes in each experiment is compared with that consumed by the group. The difference which Schuett and I have found between grouped and isolated non-schooling fishes is not obtained here. In fact, with the normal catfishes the members of groups consumed on the whole more oxygen than did isolated individuals but the difference is not statistically significant. In the case of the blinded fishes the isolated individuals consumed very little more oxygen, but here again the difference is not important. The results of three typical experiments are given in Table IV.

TABLE III

Total oxygen consumption for one hour of four grouped and four isolated catfishes, blinded and normal (expressed in cc. oxygen per liter)

	Normal		Blinded	
	Isolated	Group	Isolated	Group
1			.56	.20
2	.79	.81	.40	.57
3			.41	.34
4	1.16	1.03	.80	.63
5	.47	.67	.95	.49
6	.64	.64	.42	.49
7	.62	.66	.67	.51
8	.65	.68	.68	.68
9	.26	.42	.73	1.03
10	1.02	.98	1.12	1.27
11	.80	.74	1.00	1.01
12	.72	1.11	1.29	1.02
13	.40	.70	.56	.72
14	.88	.48	.75	.50
15	.31	.54	.48	.42
16	.88	.55	.93	.51
17	.34	.61	.23	.61
18	.77	.55	.59	.39
19	.39	.50	.54	.36
20	.87	.88	.72	.88
21	.63	.63		
22	.46	.57	.59	.53
23	.48	.45	.69	.86
24	.20	.45	.55	.53
25	.18	.41	.46	.42
	Mean difference 0.05087 cc. more oxygen for groups. Probability 0.2158.		Mean difference 0.05 cc. more oxygen for isolated fishes. Probability 0.2898.	

The cause of the discrepancy from Schuett's and my results with goldfishes is not difficult to find, nor do these indifferent results offer any contradiction to his. With normal catfishes the isolated individuals remain quiet during practically the whole experiment. In the groups, on the other hand, the fishes are in motion most of the time, being stimulated by the presence of the other individuals. Thus the rate of respiration of the grouped fishes is increased and the total is higher than for the isolated fishes. The fact that the difference is no greater probably indicates that if the factor of movement could be eliminated with catfishes a lower rate of respiration for the groups would be found than for the singles, in agreement with Schuett's work on goldfishes, where problems connected with relative motion were apparently not involved. With the blinded fishes both isolated and grouped individuals are in motion all the time, as are goldfishes, but more actively so. Here the difference is in the same direction as in the case of goldfishes, but is less and not as consistently positive. This is not surprising with the amount of motion occurring with these blinded fishes.

Table IV

Results of three experiments, showing oxygen consumption of isolated and grouped fishes, blinded and normal

Experiment	Normal		Blinded		Control
	Isolated	Group	Isolated	Group	
18	.23		.19		
	.09		.16		
	.18		.07		
	.27		.17		
	.77	.55	.59	.39	.04
19	.10		.07		
	.12		.13		
	.14		.15		
	.03		.19		
	.39	.50	.54	.36	−.04
20	.14		.28		
	.13		.03		
	.34		.18		
	.26		.23		
	.87	.88	.72	.88	−.06

It was hoped that contact responses which gave as much satisfaction as they apparently do in catfishes might produce a difference in the respiratory rate, and that this might serve as a method of determining more accurately the rôle of the different sense organs in the social behavior of these fishes. So far this has not been possible, but the results do indicate an interesting difference between the respiratory rate of schooling and non-schooling fishes; at least in these schooling fishes, such as *Ameiurus melas*, individuals stimulate one another to activity by sight or touch and thus offset or counteract the lower respiratory rate found among groups of non-schooling fishes, so that there is no significant difference in oxygen consumption between grouped and isolated individuals. With blinded fishes it may be that contact stimulation is effective in raising slightly the respiratory rate of the group so that here a significantly lower rate was not obtained.

Summary

Catfishes showed no discrimination by contact between a scoured stone and an inactive catfish, nor between a paraffined and non-paraffined stone, but gave a negative reaction to models of paraffin mixed with India ink.

There is no evidence for a gustatory element in the stimulus which one catfish receives from another. A gustatory stimulus acts only to produce a negative reaction when some unfavorable chemical is present.

The positive responses which blinded catfishes give to one another in passing are eliminated by isolation for 20 days, but reëstablished in a few minutes when the fishes are placed together.

Touch responses by blinded catfishes are positive immediately after isolation when contact is gentle enough to prevent shock.

The reactions of blinded fishes to one another due to a response to water vibrations, may be a conditioned response developed after the loss of eyesight, and is probably not effective among normal fishes.

The sight response of normal fishes to one another was not completely eliminated in all individuals by 161 days of isolation, although it was much less marked. It was reëstablished in the course of a few minutes, usually soon after contact occurred. This sight response may be instinctive but is probably subject to modification by conditioning to some extent at least.

A satisfaction evidently accrues to the catfishes from the mutual contact and pressure of the aggregations, and the importance of the thigmotactic response in these reactions is emphasized by these observations.

A comparison of the respiratory rate of catfishes, both normal and blinded, for a period of one hour gave no significant difference between the grouped and isolated individuals.

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MECHANISM OF MOVEMENT OF EPIDERMIS, ESPECIALLY ITS MELANOPHORES, IN WOUND HEALING, AND BEHAVIOR OF SKIN GRAFTS IN FROG TADPOLES

EARL H. HERRICK

Zoölogical Laboratory, Harvard University

Probably no other animal tissue is more active than an epidermis in repairing lost or damaged parts. The process is not primarily one of growth. A deficiency, either large or small, in the epidermis is rapidly covered by centripetal movement of the surrounding epithelium, and later, by reorganization and growth, the original condition is restored. Rand (1915) in considering the wound reactions of actinians says (p. 207): " In general, an epithelium will not tolerate a free edge. When such an edge arises, accidentally or otherwise, the epithelium extends until, if possible, the free edge meets and unites with some other portion of the same layer or with another epithelium. The essential function of an epithelium is to cover a surface continuously."

The investigations of Fraisse (1885), Barfurth (1891), Born (1896), Morgan (1901), Loeb and Strong (1904), Rand (1905), Eycleshymer (1907), Matsumoto (1918), Loeb (1920), Arey (1925), and Collins and Adolph (1926) point unanimously to the conclusion that wounds are at first covered by movement of the surrounding epithelial cells and that proliferation occurs later to restore the original thickness of the layer.

The cause and mechanism of the cell movement are not so well agreed upon. Born (1896) believed that the cells flatten to cover a larger surface than previously. Rand (1905), however, referring to wound healing in earthworms, says (p. 46): " There certainly is little evidence in favor of supposing that the concentric advance of the epidermis is due to the tendency of the individual cell to spread itself over the greatest possible surface."

Barfurth (1891) proposed that, while the movement of the epidermis might be in part a passive " Verschiebung " due to relief from lateral pressure in the layer, it was in the main an active movement of cells which had become " embryonal beweglich (amöboid) " (p. 417). Oppel (1912) described epithelial movement as an active movement—often a " Massenbewegung "—resulting from change of form of the epithelial cells which, however, are not ameboid. On the contrary, Holmes (1914), observing amphibian epidermis in tissue culture, decided that epithelial movement is not a " Massenbewegung " but is the result of essentially ameboid movements of individual cells. And, again, Collins and Adolph (1926) concluded that wound closure is accomplished by a mass movement or " pushing in " process of the epithelial layer and " not by the independent migration of cells " (p. 491). Loeb and Strong (1904, p. 282) considered it probable that " tension, either previously existing or called into play by the wound, is the cause " of the closure of the epidermal wounds.

Morgan (1901, p. 70), describing wounds in very young tadpoles, stated that " the wound is covered not by individual cells wandering over the exposed surface, but by a steady advance of the smooth edge of the ectoderm toward a central point. . . . As there are no muscle fibres present . . . , the result cannot be due to muscular contraction. . . ."

The artificial cultivation of epithelial tissue has given striking evidence that the cells are motile and their movement ameboid. This was suggested at an early date by Peters (1889), who studied wound closure in the cornea of frog eyes. Tissue culture studies have been carried out by Loeb (1902 and 1912), Harrison (1910 and 1914), Holmes (1913 and 1914), Oppel (1913), Uhlenhuth (1914 and 1915), Hooker (1914), Matsumoto (1918), Maximow (1925), and others. For the most part these investigators agree that movement of epithelial cells over a wound surface is ameboid and usually stereotropic.

The cause initiating the cell movement is not definitely known. Rand (1905) suggested a wound stimulus: " The most important factor in the earlier part of the process of reparation is cytotaxis; the individual columnar cells of the existing epidermis are affected by some directive stimulus and respond by an active migration, which results in the covering of the surface of injury by a protective epithelium—the first definitive step toward regeneration " (p. 52). Taube (1923) concluded that materials of injured cells flow over wound surfaces and serve as a means of chemical stimulation for the uninjured cells at the edge of the wound, causing them to migrate and later to divide. Maximow (1925) suggested that regenerative proliferation may be the result of direct stimulation by the injury or of the action of specific chemical substances. The trend of recent conclusions is in the direction of the activation of epithelial cells by a wound hormone or wound stimulus of some sort.

In the following pages are reported studies upon the skin of tadpoles of *Rana clamitans*. The studies were carried out with a view to further understanding the movement of epidermal cells over a denuded area, the behavior of host tissue in relation to a skin graft, and especially the mechanics of radial arrangement of epidermal pigment cells around a wound.

The animals used in these experiments were collected from ponds or streams near Cambridge, Massachusetts. They were usually 70 to 80 mm. in length.

The Mechanics of Wound Closure

It has been frequently noted that epidermal wounds close very quickly. In tadpoles, skin wounds 5 to 10 mm. across may be entirely closed in from 6 to 24 hours. Upon the occurrence of a wound, a quantity of blood always flows into the area and there coagulates. The coagulum usually nearly fills the wound temporarily, being about equal in thickness to the skin. Continuous microscopical examination, immediately following the operation, revealed that the edge of the epidermis starts to move toward the center of the wound within a few minutes after the wound is made. The epidermal layer advances over the coagulum, moving equally from all sides toward the center of the wound. If the edge of the wound is irregular, the advancing layer is correspondingly irregular in outline. Over large wounds the advancing epidermis thins out until it may be only one or two cells in thickness. The thinning may extend several millimeters distant from the wound, depending on the size of the wound. Figure 1 shows the limit of the thinned region of the layer and the transition to the normal condition which in this case is about 5 mm. from the edge of the rather large wound. The advancing cells are quite undifferentiated. Those of the

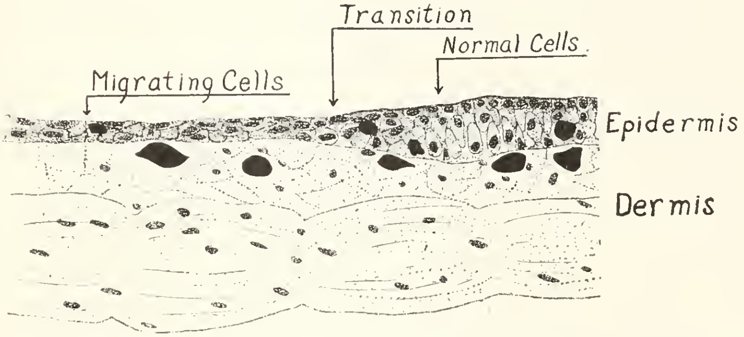

Fig. 1. Section perpendicular to the surface of skin near a wound, showing migrating cells and the transition to those not migrating. The wound was a short distance to the left of the cells illustrated.

cuticular layer are hardly distinguishable from those of other layers of the epidermis.

Sections parallel to the surface of the layer were cut from normal epidermis (Fig. 2) and from areas adjacent to a closing wound (Fig. 3). In such sections the normal cells exhibited fairly regular polygonal outlines while in an area near a closing wound the cells were from two to four times as long as wide, with the elongation in the direction of movement. This elongation of cells occurs at all points between the edge of the advancing layer and the region of transition to the undisturbed epidermis. Several writers have suggested that epithelial cells elongate perpendicularly to the edge of a wound, but an extensive reading of the literature discovers no mention of appropriate sections having been made to establish the fact. Sections perpendicular to the surface merely suggest the possibility of elongation.

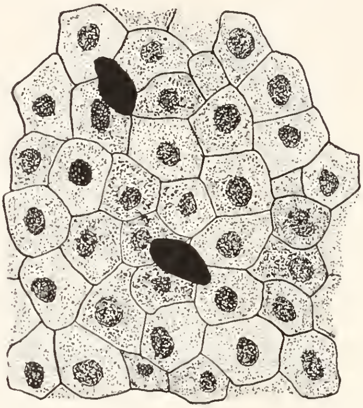

FIG. 2. Section parallel to the surface of normal epidermis. The solid black areas represent unexpanded, epidermal melanophores.

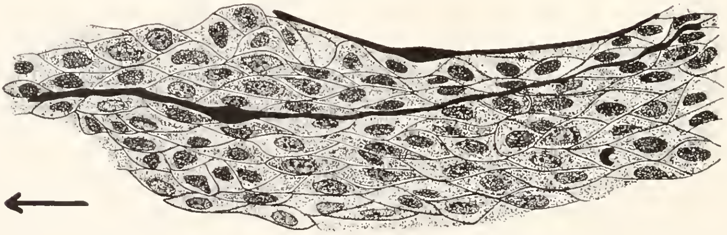

FIG. 3. Section parallel to the surface of epidermis near a wound, showing cells elongated in the direction of movement. The cells are moving in the direction of the arrow. The elongate black areas represent expanded, epidermal melanophores.

When advancing edges of epidermis meet at or near the center of the wound, there often takes place a piling up of cells. This piling up suggests that the causal factor for migration continues to operate for a time after the advancing edges have come together. After the wound has been covered by a layer of epithelium, the coagulum disappears. Within two or three days mitosis begins and the layer over the wound continues to increase until it is somewhat thicker than the epidermis in other parts of the body. Also the deficient epidermis surrounding the wound is built up, by cell proliferation, to the normal thickness of the layer.

In wounds made entirely through the skin the dermis is slow in closing, but the epidermis closes as usual, subsequently thickening until it is much thicker than the normal epidermis. Sometimes the thickness of the layer over a healed wound may be equal to the combined thickness of the normal epidermis and the dermis. When transplantations are made, the small gaps that necessarily exist between host skin and graft are closed by epidermis which grows in to fill the deficiency as if to maintain a smooth surface to the body. As the dermis is built up, the epidermis returns to its usual thickness. The function of epithelium may be not only "to cover a surface continuously," as declared by Rand, but also to restore a smooth external surface after small injuries.

Skin Grafts and the Compatibility of Tissues

The diversely pigmented integument of amphibians, possessing extraordinary capacity for repair and regeneration and readily amenable to grafting operations, offers many obvious advantages for the study of problems in tissue compatibility.

A patch of ventral, unpigmented skin from a tadpole was transplanted to the back of the same animal. Twenty-six autotransplants of this kind were made. In each instance the transplanted piece was 5 to 7 mm. square. Several operations were performed in which a dorsal patch of skin was merely cut loose and then reimplanted where it had previously been. Other patches were rotated through 90° or 180° and reimplanted.

The epidermis of an autotransplant united with that of the host immediately after the graft was made, but after that union had been effected there was no further movement on the part of the host tissue. In contrast to what happened in homoiotransplants, there was no invasion of the graft by host tissue. There was no sign of incompatibility between the ventral and dorsal skin tissue. Once the epidermal layers had united, the regenerative activity ceased. Dorsal pigmented patches which were rotated or reimplanted in their original positions were not disorganized and in a few weeks the limits of the grafts could not be recognized. The half-white, half-pigmented, lateral patches which were rotated through 180° remained unchanged, the light area of the patch remaining unpigmented and the pigmented end retaining its pigment with none spreading into the surrounding white area (Fig. 4). Animals bearing this type of graft were observed for as long as 80 days. Collins and Adolph (1926), however, observed disorganization of rotated skin grafts in experiments on *Triturus*. It seems clear that no incompatibility exists between dorsal and ventral skin of an individual tadpole, although, in respect of pigment, they are locally specific.

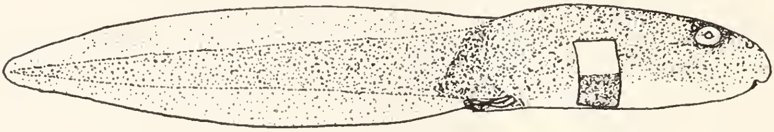

FIG. 4. Frog tadpole showing lateral, rotated graft.

Homoiotransplants were made by grafting ventral unpigmented skin from one animal onto the back of another. The grafts healed in place very rapidly so that in a few hours a microscopic inspection of the tissue in the region of the union would scarcely reveal just which were host and which were graft cells. Not all of the movement, however, in covering such gaps is accomplished by the host epidermis, for that of the patch moves almost as rapidly until the two unite. The dermal layers of host and patch are much slower in uniting than the corresponding epidermis, requiring many days or even weeks.

Sixty homoiotransplants were observed. Without exception they became occupied by host epidermal cells; and, if the animals lived long enough, dermal cells entered the unpigmented area. The grafts began to become pigmented in from 1 to 24 days after transplantation, the average being 9. The host epidermis, carrying the epidermal pigment cells, moved centripetally into the graft quite equally from all sides. Once having started, an average of 6 days elapsed before the pigment-bearing host epidermis reached the center of the patch. From a cursory examination of the surface, it was believed that the host epidermis grew over the patch, covering the epidermis as a whole. A study of microscopic sections, however, gave no indication of overgrowth or undergrowth of the epidermis, in accord with the account given by Cole (1922). The epidermis of the patch evidently undergoes destruction and is simultaneously replaced by that of the host. What appeared to be the remains of disintegrated graft cells were found in the cells at the edge of the advancing host epidermis, suggesting phagocytosis. That epidermal cells may be phagocytic at times has been stated by Loeb (1902, 1912).

The invasion of a graft by host epidermis is essentially a process of delayed wound healing. The delay is necessitated by the presence of the graft epidermis. Except for complications occasioned by the removal of the patch epidermis, the behavior of the host skin in relation to the homoiotransplant is nearly like that in ordinary wound closure. The host cells near the patch become elongated in the line of movement just as in wound healing (Fig. 3).

Fourteen transplantations were made in which patches of dorsal skin were transferred to wounds on the ventral surface of the animal. Whether the graft was an auto- or a homoiotransplant, healing took place as usual, but in the course of a few days the patch showed signs of degeneration and subsequently sloughed off until all or the greater part was lost. It is believed that this condition was not necessarily the result of incompatibility between the tissues but that the ventral side of the body, with its very thin body wall, is an unfavorable place for a graft to receive nourishment.

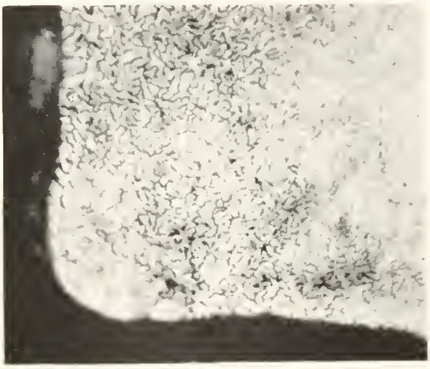

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Fig. 5. Photograph of corner of skin graft showing the network of enlarged blood vessels.

Behavior of Blood Vessels in Skin Grafts

Nearly all skin transplants early become red because of the enlargement of their blood vessels. The degree of redness varies from slight traces to a deep, solid red covering the entire patch. The reddening begins as blood vessels here and there over the patch become enlarged for very short distances. The enlarged regions then extend until a close network of such vessels may be seen over any portion or all of the patch (Fig. 5). The redness becomes apparent usually the second day after the graft is made, but it has been observed as early as 18 hours after an operation, with definite circulation in the enlarged vessels at 24 hours. The redness may persist for one to two weeks. In the greater number of cases the blood vessels enlarged until, in a few or many places in each graft, they broke to release blood into the loose dermal tissues, causing the appearance of a continuous layer of blood. This stage persisted usually less than a week. As it cleared, blood vessels of normal size could be seen, suggesting repair of the vessels. Since the vessels that occasioned the temporary redness became filled with blood within the short time of a few hours after the operation and because their arrangement is so similar to that of the vessels normally present in the ventral skin, it is probable that they are not newly developed after transplantation, but are the original vessels of the grafted skin.

Cole (1922) has described the reddening of homoiotransplants of tadpole skin but states that autotransplants do not show this reaction. He says (page 391): "The occurrence of such a process is evidence of a rather violent chemical reaction going on between the protoplasms of the graft and host, and a merely specific difference between the two could set up the reaction." Cole's conclusions, however, are not in accord with the experiments described in the present paper, for in these experiments both auto- and homoiotransplants became reddened in nearly all cases. Since white autotransplants never became pigmented and since there was no other indication of any reaction between tissues, it seems evident that increased vascularity is not an indication of incompatibility between tissues. Furthermore, there is considerable evidence that enlargement of the blood vessels is due to mechanical stretching of their walls. As circulation becomes established in the graft, the blood exerts pressure on the walls of the vessels. In normal tissues, tonus of the vessels balances this internal pressure. There is slight variation in the caliber of normal vessels to meet the varying needs of the animal, the tonus being controlled by the nervous system. In transplanted patches the nervous connections have been cut during the operation, leaving the blood vessels of the patch without normal tonus, only a certain elasticity intrinsic to the vascular tissue itself persisting. It is highly probable, therefore, that enlargement of blood vessels following transplantation is due to stretching of the vessels from internal pressure in the absence of nervous control and not to a specific reaction between tissues as concluded by Cole.

May (1924), after transplanting lizard (*Anolis*) skin, concluded (p. 553): "Transplants of pigmented skin lose their colour-changing power immediately on being completely disconnected from neighbouring tissues, and regain it slowly as nerves regenerate." It has not been ascertained just when nerves regenerate to innervate the grafted tadpole skin, but the apparent resumption of control of the blood vessels agrees in time with that given by May for the regeneration of nerves in the skin of *Anolis*. May's conclusions apply very well to the apparent loss and regaining of nerve control of the blood vessels of the tadpole grafts.

Radial Arrangement of Epidermal Pigment Cells Around a Wound or Skin Transplantation

When expanded epidermal pigment cells lie upon or near a skin wound which is healing or a transplanted patch of skin which is becoming pigmented, they show a particular arrangement in relation to the wound or graft. In general, the axes of elongated melanophores are perpendicular to the edge of the wound or, in other words, they are parallel to the line of movement of the epidermal cells toward the wound or graft. In any one localized area the greater number of epidermal melanophores are parallel (Fig. 6). Some cells may be at an angle to others, or occasionally a melanophore may be perpendicular to the axes of the greater number, but such cells are too few to impair the conspicuous radial arrangement of the majority. This arrangement may extend for several millimeters external to the edge of the wound or graft. The area over which radial arrangement may take place was found to coincide with the extent of the region within which migration

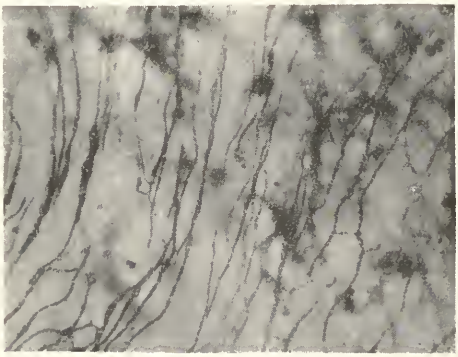
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Fig. 6. Photograph showing the nearly parallel arrangement of epidermal pigment cells near a wound.

of the epidermal cells occurs. In Fig. 1 the limit of radial arrangement occurs at the point labeled "transition." This observation is very significant in relation to the mechanics of the radial arrangement.

It is obvious that radial position of a pigment cell will not be evident unless the cell is expanded. Radial arrangement depends upon expansion of the melanophores, but the causes of radial arrangement and expansion were found to be distinct.

Since investigation of the causes of radial arrangement could be carried on only when the epidermal melanophores were expanded, it was desirable to find an artificial means of producing expansion. The following technique was used. An injection fluid was prepared by adding desiccated bovine pituitary gland to Ringer's solution and filtering the mixture through filter paper. The powdered gland material dissolved and filtered better if the liquid were slightly warm. About one gram of desiccated gland was used in 10 cc. of Ringer's solution, but wide variations of this concentration proved to be effective. Intraperitoneal injections were made, using a small hypodermic syringe with a dosage of about 0.2 cc. After an injection of this kind the melanophores began to expand in about twenty minutes and continued expanding for one or two hours until maximal or nearly maximal expansion was reached. Allen (1917), Swingle (1921), and Collins and Adolph (1926) have studied in considerable detail the effect of pituitary gland and its extracts on pigment cells, but in the experimental work now being described the pituitary extract was used merely to expand melanophores in connection with the study of the problem of their radial arrangement.

Expanded epidermal melanophores near a wound which was being covered, or around grafts toward which epidermis was moving, exhibited more or less marked radial arrangement. Therefore radial arrangement of melanophores may be expected wherever there is a migrating epidermis whose melanophores are expanded. After a wound or graft has been covered by the surrounding epidermis and the layer has nearly returned to its normal thickness, the melanophores begin to lose their special arrangement. In homoiotransplants radial arrangement persisted from one to two weeks or even longer. In one large homoiotransplant, after it had become pigmented and the radial arrangement lost, two holes, about 3 mm. apart and each about 1 mm. across, were cut through the skin of the graft. The pigment cells were partially expanded. Twelve to eighteen hours afterward there was marked radial arrangement around each of the holes.

In considering the mechanism of radial arrangement of melanophores, the conditions of both normal and migrating cells must be kept in mind (Figs. 2 and 3). During the migration of the epidermal cells, their space relationships are continually changing. Further, *the epidermal cells closest to the edge of the wound or graft move faster than those successively farther from the wound or graft.* In the initial advance of cells in covering the wound, the cells closest to the edge of the wound travel a greater distance than cells successively farther from the edge. It is believed that the mechanism of radial arrangement may be completely described from the facts just stated.

Unless a pigment cell is lying with its long axis exactly perpendicular to the line of movement of the epidermal cells, the end of the melanophore nearer the edge of the wound will be carried faster than the remainder of the melanophore. The carrying of one end of the cell faster than the other in a given direction will tend to rotate the comparatively long pigment cell to lie in a new position, more nearly in the line of movement of the epidermal cells. Since a single epidermal melanophore may be as long as the diameters of twenty epidermal cells, this action could easily occur. Continuous microscopic observations made on living epidermis at the edge of a wound support in every detail the suggestion just made. Such observations were made continuously on a single wound for as long as twelve hours.

Suggestions for apparatus used in making the observations just mentioned were obtained from Clark (1912). A small glass box was constructed, part of one side of which was made of " cover slip " glass to permit the use of high-power microscope objectives. The box was filled with paraffin to a depth of about 5 mm. The tube of an ordinary compound microscope was turned to the horizontal position and the glass box was secured to the mechanical stage. With the box in this position, the open side was uppermost and the side made of the thin glass was next to the objective lens of the microscope.

When a wound was to be studied, the tadpole was first anesthetized by placing it in water to which had been added a few drops of ether. As soon as anesthesia was produced, the animal was placed in the glass box, which contained a 0.05 per cent chloretone solution. Chloretone is a very slow-acting anesthetic and causes melanophores to expand. Ether was used as described merely to hasten immobilization of the animal before the melanophores could expand. The animal was held against the thin side of the box by means of pins thrust into the paraffin.

Usually transmitted light from the mirror or directly from a lamp was used, but reflected light with or without transmitted light was sometimes used. Sufficient light could be transmitted through the tail of a tadpole to permit the use of an oil-immersion lens, but the best results were obtained by means of a 4 mm. lens and a 10 × to 18 × ocular. Magnifications up to nearly 1000 diameters were frequently obtained. The animals remained alive in the glass box for over 24 hours, so that very satisfactory records were obtained up to that length of time.

A vertical drawing board was used to permit the employment of a camera lucida. Individual pigment cells were located and followed for several hours. Hence by making drawings at frequent intervals, the exact courses of the cells could be followed. The movement was too slow to be observed except by means of a series of drawings.

In following the movements of melanophores, the neighboring epidermal cells were observed at the same time. It was found that the melanophores did not orient themselves irrespective of epidermal cells but moved with them and at approximately the same rate. Several investigators have concluded, from tissue culture studies, that melanophores possess the power of self-movement. The above described observations afford no ground for doubting that melanophores do have the power of self-movement, but there is every reason to believe that, under the conditions of the experiments in question, they are no more active than the other epidermal cells and that their radial arrangement depends upon the movement of the epidermal cells and not upon the activity of the melanophores themselves. Figures 7 and 8 are series of camera lucida drawings of groups of pigment cells which were near

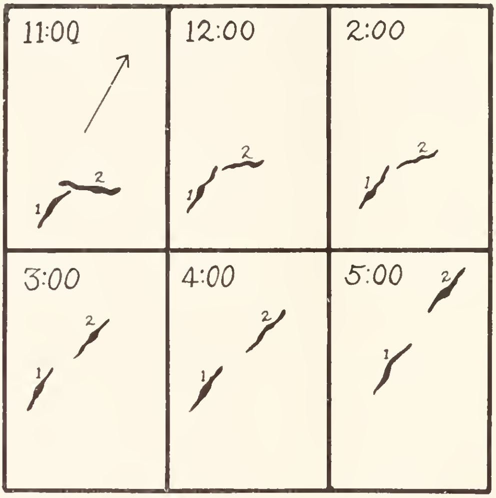

Fig. 7. First section of series of camera lucida drawings showing the paths taken by epidermal melanophores as they are carried toward a wound, coming to lie with long axes nearly perpendicular to the edge of the wound. The arrow indicates the direction of movement.

healing wounds. The drawings show the successive positions of the melanophores as they became more nearly perpendicular to the edges of the wounds. The time intervals between successive drawings are indicated.

With regard to radial arrangement of pigment cells, there was a striking difference between auto- and homoiotransplants. There was never extensive radial arrangement around autotransplants. This is not strange, however, since in autotransplants there is little or no migration of epidermis after the epidermis of the host meets that of the graft. In fact, this is further evidence in support of the proposed explanation for radial arrangement.

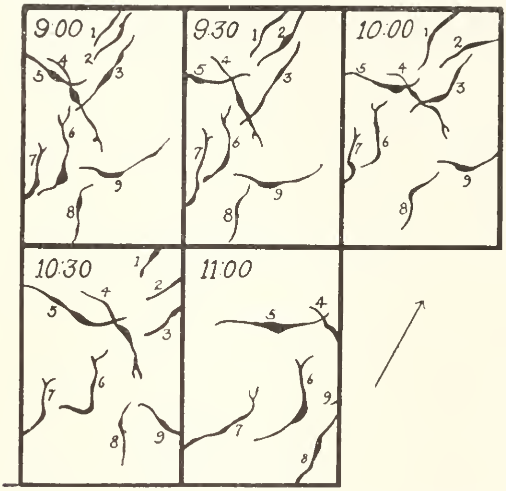

FIG. 8. Second section of a series of camera lucida drawings showing the paths taken by epidermal melanophores as they are carried toward a wound, coming to lie with long axes nearly perpendicular to the edge of the wound. The arrow indicates the direction of movement.

The writer wishes to express his sincere appreciation to Dr. H. W. Rand for his advice and aid throughout the period of this work.

SUMMARY

1. Immediately following the occurrence of wounds in the skin of tadpoles of *Rana clamitans,* the surrounding epidermis moves to cover the area. Wounds 5 mm. across may be covered in from 6 to 24 hours. The advancing epidermis is reduced to one or two cells in thickness, thus covering a larger area than previously. The epidermal cells become considerably elongated in the direction of movement. After the

advancing edges have met, the cells resume their previous shape. Subsequent mitosis restores the original thickness of the layer. There is considerable evidence for stereotropism in epidermal migration.

2. In wounds which cause a deficiency in the dermis, the epidermis thickens over the wound area until the smooth contour of the body-surface is restored.

3. In the region surrounding a healing wound there is temporarily a deficiency in the number of epidermal pigment cells due to their movement into the wound.

4. Autotransplants of white ventral skin transferred to the pigmented backs of tadpoles retained their specificity. They remained unpigmented for more than 100 days. Lateral and dorsal autotransplants in which the patches of skin were rotated through 90 or 180° also retained their specificity. There was no change in the pigmentation or the cellular structure of such grafts.

5. In all cases where homoiotransplants of white ventral skin were transferred to the pigmented backs of tadpoles the region of the graft became pigmented. Epidermal pigment appeared at the edge of the graft in an average of 9 days after the graft was made, and in the course of about 6 more days had extended to the center of the patch. Dermal pigment entered the graft area many days after the epidermal pigment had completely covered the patch.

6. The initial pigmentation of grafts results from a replacement of the graft epidermis by that of the host. The pigment cells are carried along by the host epidermis as it covers the patch. There is considerable evidence that the graft epidermis is destroyed by phagocytic action of the host epidermis. However, the epidermal layers of graft and host always unite immediately after the graft is made, indicating some initial affinity between the two layers.

7. Patches of skin which were grafted onto the ventral side of the body always degenerated. The ventral side of the body is perhaps an unfavorable place for grafts to receive nourishment.

8. The replacement of the epidermis of a graft by host epidermis is, essentially, a process of delayed wound healing.

9. In nearly all cases grafts, soon after transplantation, became reddened as the result of an enlargement of the dermal blood vessels of the graft. There is considerable evidence that the enlargement of the blood vessels is due to blood pressure which mechanically stretches the vessels in the absence of the normal tonus, the nerve connections having been cut at the time of transplantation. Later, and probably in consequence of restoration of nerve connections, the dermal vessels became reduced to normal proportions.

10. Radial arrangement of the epidermal melanophores occurs around homoiotransplants and skin wounds—that is, wherever there is translatory movement of the epidermis. In any migrating epidermis in which the melanophores are expanded, their long axes tend to become parallel to the direction of migration. The position of the individual melanophore, in radial or parallel alignment, is a consequence of the movement of the epidermal cells near a wound and is not due to independent orientation of the melanophores. The epidermal cells nearest the wound move first and most rapidly, therefore tending to rotate the elongated melanophores into the line of movement of the epidermis. Since the movement of the epidermis is centripetal, the melanophores become arranged radially around the point toward which the epidermis moves.

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OXYGEN DEFICIENCY AND SEWAGE PROTOZOA: WITH DESCRIPTIONS OF SOME NEW SPECIES [1]

JAMES B. LACKEY

(From the Sewage Investigations Laboratory, New Brunswick, N. J., and Southwestern College, Memphis, Tennessee)

It is a common observation that the protozoa of hay infusions and other liquid media generally seek the top of the culture where oxygen is to be found. Investigation of a rich culture either in a jar or on a slide shows that only a few inhabit the oxygen-poor regions: *Metopus* among the ciliates; some of the very small flagellates and small amoebas, mostly of the limax type. Anaërobic protozoa are well known. Juday (1919) has described a freshwater anaërobic ciliate; Lauterborn (1908) has discussed a number of species, many of them bizarre forms, from the oxygen-poor waters of the Rhine. Lackey (1925, 1926) has listed twenty-nine species common in sewage containing little or no dissolved oxygen, but often abundant H_2S or CO_2; while Cole (1921) has discussed the oxygen supply of animals living under such conditions.

Most of the protozoa inhabiting sludge tanks are small and do not occur in great numbers—except those which live at the very top and are not considered in this paper, for they have an abundance of oxygen and may be transient forms in the tanks as well—so that they are not often noted by students unless a careful search is made. All of those ciliates characterized by Noland (1925) as living in water whose oxygen saturation is below 45 per cent have been encountered at one time or another, but usually in small numbers, with the exception of *Metopus sigmoides*. As a result of examining the protozoan fauna of the digestion tanks of five New Jersey towns, the Passaic River in some of its most polluted stretches, and septic tanks from three locations in Tennessee, it has been found that the same small group of protozoa is to be found in each of these situations.

Of the forms listed previously (Lackey, 1925) two ciliates and one flagellate were described as new species. The present paper includes four new flagellates and two new rhizopods. The wide distribution of this group argues that the factors limiting their occurrence are very largely those of anaërobism, for such conditions as pH, temperature, dissolved gases, and food substances must have varied widely in the several locations examined. Sufficient check has been made on the first two of these conditions in sludge digestion to make this assumption plausible, and it is known that they certainly vary from time to time, and that the amounts of CO_2 and H_2S fluctuate considerably also in any given tank, apparently without a corresponding fluctuation of the protozoan fauna as a whole, in the same tank. It is also certain that while all sewage contains decomposition products which can possibly serve as food for the organisms, the variety of these substances is too great to be a probable limiting factor.

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[1] Journal Series Paper N. J. Agricultural Experiment Station, New Brunswick, N. J. Dept. Sewage Research.

To ascertain the effects of dissolved oxygen on two protozoa characteristic of sewage disposal plants, tall cylinders were set up, so that compressed air could be forced through porous plates in their bases. This allowed aëration of the sewage (eventually producing activated sludge) or kept the sewage under completely anaërobic conditions. *Opercularia* sp., a large peritrichous holozoic ciliate and an active bacterial feeder, was selected as the obligatory aërobe, for it is abundant in the trickling filters, but never occurs in its active state in the tanks. *Trepomonas* was selected as the obligatory anaërobe, for it is characteristic of the depths of the tanks. Table I shows the behavior of these two forms in the cylinders under conditions varying from no aëration to constant aëration. Preliminary examination of the sewage at the treatment plant showed 3300 *Trepomonas* and 500 *Opercularia* per cc., and after standing in a refrigerator 24 hours there were 2900 *Trepomonas* per cc., and no active Opercularia. It will be noted that the sludge when examined at the disposal plant contained both active *Opercularia* and *Trepomonas.* There was only a trace of oxygen present. The sample was taken to the laboratory and put in the refrigerator and 24 hours later contained no dissolved oxygen. Its protozoan population had changed in this time; all colonies of *Opercularia* contained only closed-up individuals.

After 6 hours aëration 65 per cent of the *Opercularia* were active, whereas with no aëration none of the *Opercularia* were active. No count was made, but the normal population of *Trepomonas* seemed to be present. Under aërobic conditions, however, the 6 hours had reduced the numbers of *Trepomonas* almost 96 per cent. Many dead or apparently dead ones were seen. Their protoplasm was greatly vacuolated and the cells sometimes much above normal size.

Twenty-four hours of aëration was sufficient to cause them to disappear and they never reappeared in this sample. All the *Opercularia,* on the contrary, became active and showed an increase in numbers for 24 hours, then a gradual decrease. When the experiment was discontinued after 196 hours, there were few protozoa of any sort in either aërated or unaërated sewage, but most of the *Opercularia* were active in the aërated, although they presented a rather starved appearance, due probably to disappearance of their food.

Although the results practically speak for themselves, supporting evidence is easily obtained. During an examination of settled sludge from the Chatham, N. J., activated sludge plant, on August 2, the sampling showed no dissolved oxygen and there were present 9200 *Trepomonas* per cc., and only about 260 *Opercularia* per cc. The *Opercularia* were all closed up, inactive, while the *Trepomonas* were swimming about vigorously. This sample was then aërated for 3 hours, when an examination showed that all the *Opercularia* were actively feeding while about half the *Trepomonas* were killed and half active.

TABLE I

Numbers of Organisms Present With and Without Aëration
(Numbers refer to active organisms throughout)

TIME	AËRATED		UNAËRATED
		No. per cc.	No. per cc.
After 6 hours	Trepomonas	140	No count
	Opercularia	3200	0
After 24 hours	Trepomonas	0	3300
	Opercularia	12600	0
After 48 hours	Trepomonas	0	4500
	Opercularia	9300	0
After 120 hours	Trepomonas	0	5400
	Opercularia	8300	0
After 148 hours	Trepomonas	0	4000
	Opercularia	4500	0
After 172 hours	Trepomonas	0	2000
	Opercularia	4500	0

At the end of 6 hours aëration there were no signs of living *Trepomonas* in this cylinder, although the *Opercularia* were thriving and numerous small flagellates such as *Bodo, Cercobodo, Pleuromonas, Monas,* and *Tetramitus* were active. After 49 hours aëration a large

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protozoan fauna was present, the small flagellates of the group listed above being 15,300 per cc., and *Trepomonas* being absent. In a control of unaërated sludge there were, per cc., 5900 *Trepomonas,* 900 *Tetramitus,* active, and perhaps 175 inactive *Opercularia.*

It is not intended to list the protozoa occurring in activated sludge or trickling filters or, in general, in sewage which contains much dissolved oxygen. But from the above experiments and similar ones, it is apparent that many protozoa encyst when swept into the oxygen-free water of a digestion tank or the bottom of a river heavily polluted with sewage; that like *Opercularia,* they are able to reduce their metabolic activities to a minimum and so survive for a time, at least, in this adverse environment. If conditions become favorable again, they excyst and become active. It is also seen that there are some forms which live under either condition and some which thrive only in the absence of oxygen. This latter factor is so sharply limiting that we find a small and cosmopolitan group of protozoa which may be regarded as common in Imhoff tanks, septic tanks, and the deeper waters of heavily polluted streams.

Table II does not include many forms which have been seen, but not sufficiently studied for proper identification. Some of these will undoubtedly prove to be new species when better known. It is therefore seen that careful investigation of this particular type of habitat will be productive of an acquaintance with a decidedly unusual and interesting group of protozoa.

Description of New Species

The following descriptions are concerned with two new rhizopods and four new flagellates, which have been found in studying the waters of sewage disposal plants and the water of a creek near Camden, N. J., which was heavily polluted with sewage. Only one of the flagellates, *Chroomonas cyaneus,* was found in the creek, the others being from sewage disposal plants. The new species are illustrated in Plate I, enlarged about 1000 times.

VAHLKAMPFIA FRAGILIS, Sp. Nov. Fig. 4.

Organism small, free-living, 5 to 15 microns long, naked, relatively abundant in some sewage, especially in cultures. Pseudopodia lobose, two to three in number, clear, steadily formed rather rapidly and always at end of cell opposite contractile vacuole and nucleus.

One contractile vacuole, always posterior to the nucleus, constantly present, emptying seldom. Nucleus always oval, flattened, with two to three large endosomal granules (chromatin masses?) in center. Nuclear membrane delicate. Several hyaline spheres of varying size, oil or albumen, located in posterior region. Endoplasm finely granular, never extending into pseudopodia.

No flagellated stage found.

Table II

Group I

Present Only in Small Numbers or Infrequency in Digestion Tanks

Rhizopoda

Dimastigamoeba gruberi
Vahlkampfia limax
Vahlkampfia guttula
Hartmanella hyalina
Vahlkampfia albida
Chlamydophrys stercorea

Flagellata

Mastigella simplex
Cercobodo longicauda
Cercobodo crassicauda
Cercobodo ovatus
Monas amoebina
Monas minima
Monas vulgaris
Anthophysa vegetans
Helkesimastix faecicola
Bodo caudatus
Bodo lens
Bodo mutabilis
Pleuromonas jaculans
Oicomonas termo
Cyathomonas truncata
Dinomonas vorax
Tetramitus descissus
Tetramitus pyriformis
Hexamitus inflatus
Clautriavia parva
Euglena gracilis
Menoidium incurvuum
Peranema trichophorum
Distigma proteus
Petalomonas mediocanellata
Petalomonas carinata
Heteronema acus
Entosiphon sulcatum
Notosolenus orbicularis
Chilomonas paramecium

Ciliata

Hexotrichia caudatum
Colpoda inflata
Glaucoma scintillans
Plagiopyla nasuta
Colpoda cucullus
Cyclidium glaucoma
Paramecium putrinum

Group II

Always Present in Absence of Oxygen in Tanks

Rhizopoda

Chlamydophrys minor, sp. nov.
Vahlkampfia fragilis, sp. nov.

Flagellata

Mastigamoeba viridis
Mastigamoeba longifilum
Mastigamoeba reptans
Mastigamoeba radiosa, sp. nov.
Trepomonas agilis
Bodo glissans, sp. nov.

Ciliata

Holophrya sp.
Metopus sigmoides
Saprodinium putrinum
Trimyema compressa

Nutrition apparently saprozoic.

Division binary in active stage. Forms very small cysts, thick-walled, with a few slight protuberances. Excysts in fresh raw sewage (by putting cover-slips in Petri dishes of old cultures, cysts are collected which can be watched on being transferred to fresh material).

Rather common in five samples obtained.

MASTIGELLA RADIOSA, Sp. Nov. Fig. 2.

Animal with spherical body, about 20 microns in diameter, free-swimming or floating. Pseudopodia much like those of *Amoeba radiosa,* occasionally branching, clear, up to 80 microns long. Single flagellum up to 100 microns long, used with a lashing or whiplike motion. One contractile vacuole. Nucleus central, spherical with endosome, membrane very thin, chromatin granules very small. Cytoplasm finely granular, no zones being present. Several types of granules such as oil spheres, or crystalline bodies, present.

Some apparent binary fission stages seen. Nutrition saprozoic. Rather rare; found in two of the tanks examined.

MASTIGAMOEBA VOLUTANS, Sp. Nov. Fig. 1.

Body elongate, flattened, 25 to 30 microns long, with a flagellum, vibratile in anterior third, slightly tapering, one and a half to two times body length. Cortical layer of protoplasm clear, hyaline, endoplasmic portion finely granular, reticulate, with numerous small, square crystals and numerous spheres of varying size. Due to the fact that they stain with methylene blue and that in some of these animals a clear vacuole was found which contained from one to three of these spheres, they are assumed to be volutin.

Pseudopodia very short, numerous, rounded.

Contractile vacuole single, in posterior part of cell.

Nucleus near anterior tip of cell, round in outline, with a central endosome surrounded by minute chromatin granules within a clear zone of nuclear sap. Membrane thin, with a rhizoplast visible in the living animal, from the flagellar insertion to its surface.

Nutrition apparently saprozoic.

Reproduction not observed.

Somewhat common in samples from six localities.

CHLAMYDOPHRYS MINOR, Sp. Nov. Fig. 3.

A very small form, showing constant differences from *C. stercorea.* Diameter of the transparent shell is 20 microns. Cytoplasm finely granular, extending out as a few finely-branching pseudopodia, which only occasionally anastomose and never become thread-like.

Contractile vacuole single, median. Nucleus centrally located, spherical, with a single endosome. A central zone of black, refringent, crescent-shaped granules is present.

Nutrition apparently saprozoic. Reproduction not observed. Movement slow.

Rather common in most sewage.

BODO GLISSANS, Sp. Nov. Fig. 6.

A somewhat flattened elongate organism, rarely over 20 microns long, cell of definite shape, but quite plastic. Two flagella, emerging on the ventral surface, anteriorly, from a lip-like fold. Swimming flagellum about body length, very tenuous and very active. Trailing flagellum slightly longer, thicker, used as an axis on which the animal glides. Forward movement rapid, path straight. When the animal changes its path, its amoeboid nature becomes evident.

No nucleus visible either in living specimens or those treated with neutral red or iodine. Some visible granules may be chromidia. One contractile vacuole, median. Protoplasm in anterior end mostly clear, but a few small spheres, probably oil, may be seen posteriorly. No visible kinetic apparatus. Reproduction by longitudinal division, while active. Nutrition saprophytic. No cysts observed. Rather common in sewage from several locations.

The animal is placed in the genus *Bodo*, following Calkins' (1926) key, but it is not holozoic, and the two flagella are not terminal but subterminal. None of the species described in Pascher's manual (1914) fit it. The position of the contractile vacuole and the inability to find an organized nucleus are also unique features.

CHROOMONAS CYANEUS, Sp. Nov. Fig. 5.

Organism pyriform, obliquely truncated anteriorly, tapering to a somewhat extended point posteriorly, never exceeding 10 microns in length. Two equal or subequal flagella emerge from a slight depression anteriorly. They are about body length, directed forward in swimming, and the path of the animal is a spiral. Two or more small contractile vacuoles are located at their base. There is no eyespot, nor can other structures be distinguished in this region. The nucleus is central and apparently there is no endosome, but only granular masses of chromatin. Chromatophores two in number, band-shaped, curved, of a bright blue color which speedily diffuses into the surrounding water when the animal disintegrates. Pellicle thin, rigid. One case of binary fission while active was noted, but no cysts were found.

These organisms were not found in sewage, but were abundant in the waters of a creek, heavily polluted with sewage, near Collingswood, N. J.

SUMMARY

There is an unusual group of protozoa to be found in the oxygen-poor and oxygen-deficient waters of sewage treatment plants.

Most of these are apparently facultative anaërobes. A few appear to be obligatory anaërobes.

It is shown that the presence of dissolved oxygen in sewage allows *Opercularia* to thrive, while the absence of oxygen is fatal to this protozoön, if the condition endures for several days. The reverse conditions are found to obtain for *Trepomonas*, except that active aëration of the water is quickly fatal.

Six new species of protozoa from sewage or polluted waters are described.

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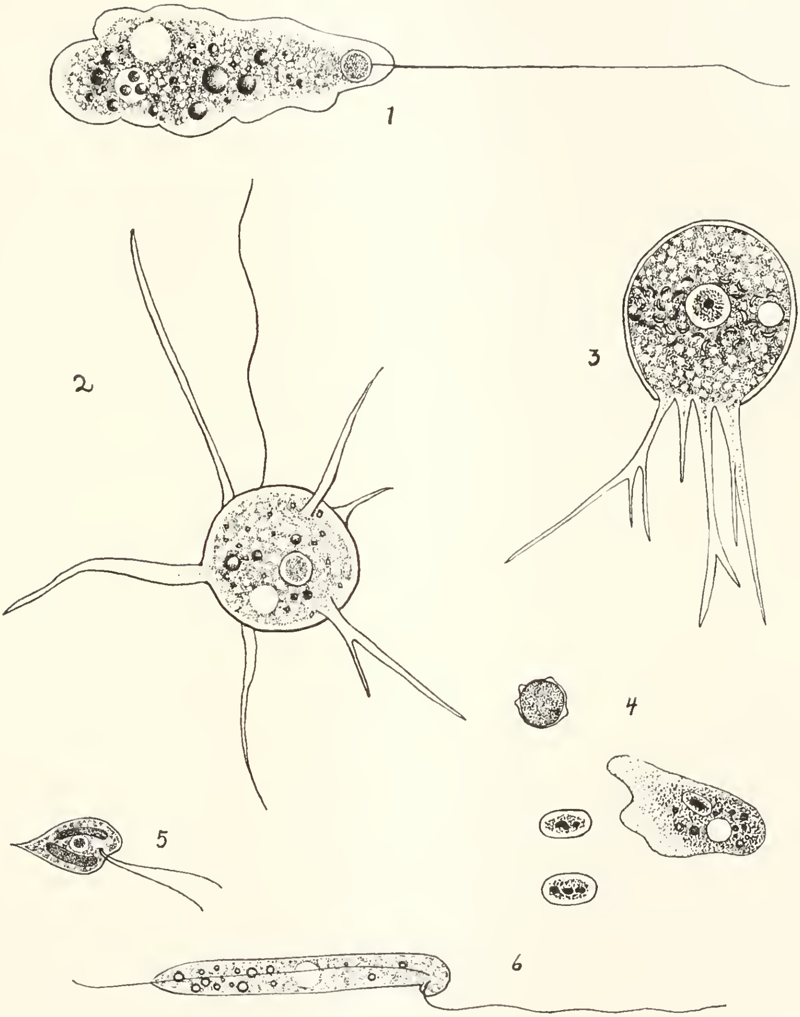

Plate I

MODIFICATION OF TRAITS IN MOSAICS FROM BINUCLEATE EGGS OF HABROBRACON[1]

P. W. WHITING

(From the Zoölogical Laboratory, University of Pittsburgh, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

Egg binuclearity has been suggested to account for the origin of a few rare mosaics in *Drosophila* and of hereditary mosaicism in *Lymantria*. Most of the mosaics which have been found in the parasitic wasp, *Habrobracon juglandis* (Ashmead), are likewise best explained by this hypothesis. The theory advanced by the writer (Whiting, P. W., 1922) is that after extrusion of the first polar body, the second oöcyte nucleus gives rise to two (reduced) nuclei which either take part in parthenogenetic cleavage (male mosaics) or segment after one has been fertilized (gynandromorphs). Post-reduction of binucleate eggs from heterozygous females would then result in mosaic males or, in case of fertilization of one nucleus, in gynandromorphs in which maternal contribution to male and female parts was different.

Mosaics in *Habrobracon* are very infrequent. The present paper is based on 132 mosaic males and 92 gynandromorphs which have occurred scattered through the cultures of various investigators. Whenever a mosaic has been reported it has been given a serial number in the "freak book" with a statement of its origin, a description, and any other pertinent data. The specimen, placed in a gelatine capsule, is then preserved in alcohol for future reference.

Mosaic Males from Heterozygous Mothers

Virgin females heterozygous for various genes have been bred in tests for linkage. Most of the mosaic males have been found in connection with these studies. They therefore arise from unfertilized eggs and are mosaic for one or more of those traits for which their mothers were heterozygous. It will be convenient first to give a record of these male mosaics as regards the various genes involved.

Females heterozygous for orange, *o* (eyes) (Chromosome I), have produced thirteen sons mosaic for this trait. When tested by mating

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[1] The investigations here reported have been aided in part by grants from the Committee on Effects of Radiation on Living Organisms (National Research Council). The drawings for Plate I have been made by Kathryn A. Gilmore.

with orange females two of these mosaics had type (black) daughters, one had orange daughters, and one had both black and orange daughters. The line of division between black and orange ommatidia is difficult to determine, showing more or less gradation. In general the orange regions of eyes of mosaics appear darker than in normal orange and ocelli contain more or less brown pigment so that it is sometimes difficult to classify them as different from " wild type." Ocelli of normal " orange " contain more or less red pigment but not brown, while those of wild type contain much dark brown pigment.

Females heterozygous for ivory, o^i, have produced sons with mosaic eyes which may be classified as follows: nineteen had compound eyes described as mosaic for orange and black; nine for pale orange and black; five had one eye ivory, the other ivory grading through orange to black (Fig. 1); one had one eye black, the other ivory grading through orange to black. Observations were made on ocelli of thirty of these. They were classified as wild type, five; more or less brown pigment, three; very little brown pigment grading to colorless, five; wild type grading to much red pigment, three; more or less red pigment, ten; completely devoid of pigment, four. Breeding tests made of ten of these showed five breeding as black, three as ivory, and two as black and ivory. Twenty showed mosaicism for other traits for which the mothers were also heterozygous.

Females heterozygous for ivory have produced sons showing no very obvious mosaicism in eye color but which were mosaic in other traits of maternal origin affecting wings, legs, or body color. One with eyes and ocelli black bred as ivory; one with ivory eyes and ocelli bred as black and ivory. One with orange eyes and a trace of brown pigment in ocelli bred as ivory. One with orange eyes and ocelli bred as black. Six had pale orange eyes among which one had a trace of brown pigment in ocelli and bred as black; one had much red and brown pigment in ocelli; one had much red pigment in ocelli; while of the other three with colorless ocelli, one bred as black and one as ivory.

Females heterozygous for ivory produced aberrant sons showing no obvious mosaicism in any trait. Eleven of these had eyes classified as orange among which two had colorless ocelli while nine had much red pigment in ocelli. One of the former and six of the latter showed by breeding test that they were actually mutants to orange and one of the latter bred as black and was therefore a mosaic. Seven had eyes classified as pale orange among which one had no pigment in ocelli, two had much red pigment, while four had a small amount of red pigment. One of the last bred as black and was therefore mosaic and one bred as black stumpy and as ivory non-stumpy and therefore had mosaic gonads.

The sixty-two exceptional males produced by females heterozygous for ivory all showed orange modification of eye color to greater or less extent except that one which bred as ivory had black eyes, and one which bred as black and ivory had ivory eyes. The entire group includes forty-four from untreated stock and eighteen from mothers X-rayed as larvæ in twelve cases and as adults in six cases. Among the seven proved by breeding test to be mutants to orange only one was produced by an X-rayed (larva) mother. The seven mutants appeared entirely normal, showing mosaicism in no respect, not even as regards body pigmentation. There is no evidence that X-radiation has caused either modification or mutation to orange.

An orange-ivory compound virgin female, $o\,o^i$, produced a male (Fig. 2) with left eye orange, right orange dorsally, ivory ventrally with well-marked line of division between the two regions. Ocelli had red pigment granules characteristic of orange. The specimen bred as ivory.

Females heterozygous for miniature, *m* (body, antennæ, wings, legs) (Chromosome I), have produced two mosaic males showing clear-cut difference in antennæ, wings, legs, and general body size.

Females heterozygous for cantaloup, *c* (eye color) (Chromosome II), have produced fourteen mosaic males. Black and cantaloup regions are distinctly marked off in these mosaics in compound eyes and frequently even in ocelli in contrast to gradation observed between black and orange or black and ivory (Figs. 3–9).

Females heterozygous for long, *l* (antennæ, wings, legs) (Chromosome II), have produced eight mosaic males, one breeding as long. Difference was clear-cut, being evident in antennæ and wings, and could even be noted in legs.

Females heterozygous for narrow, *n* (wings) (Chromosome II) have produced three mosaic sons with clear-cut difference showing in wings.

Females heterozygous for Minnesota yellow, *My* (antennal segments) (Chromosome II), have produced five mosaic sons. The character is variable, modified by temperature, but the contrast is striking in antennæ of these mosaics.

Females heterozygous for reduced, *r* (wings) (Chromosome IV), have produced twelve mosaic sons. Difference is clear-cut and any one wing is either type or reduced. Tests showed four breeding as type and one as reduced. There were likewise produced from heterozygous mothers a reduced breeding as type and a type breeding as reduced, which were therefore also mosaic.

Females heterozygous for fused, *f* (tarsi, antennæ, wings) (Chromosome IV), have produced two mosaic sons. Another male mosaic for fused, found in an inbred culture (No. 3), was in every other respect similar to Stock 3, but proved sterile in observed matings with three females. The method of origin is uncertain, no other fused were found in the culture and it is therefore possible that this may have been a somatic mutant. The combination of traits is unmistakable and this specimen showed typical fused in tarsi, antennæ and wings. Besides this case the locus has been known to mutate four times. Modification of fused in mosaics will be discussed below.

A male mosaic for glass, *g* (eyes and antennæ) (Chromosome IV), occurred in a mixed culture so that parentage is uncertain. The left antenna was very thin as in typical glass, the right type. The eyes were each mosaic with clear-cut regional distinction between glass and type (Figs. 20, 21), but the outline of ommatidia near the margin departs from the normal hexagonal form probably due to absence of pressure from other ommatidia in development. The glass regions are genetically orange, the non-glass black. There is gradation of dark pigment into the orange regions as expected and outlines of bases of abortive ommatidia are rendered visible by the presence of this pigment.

A male mosaic for wavy, *wa* (wings), and a male mosaic for broad, *br* (thorax) (Fig. 16), have each been produced from a heterozygous mother (Chromosome V).

Two males mosaic for white, *wh* (eyes) and one for attenuated, *at* (antennæ) (Chromosome VI), have been produced by heterozygous females. White regions of compound eyes and of ocelli are, as in the case of cantaloup, sharply distinct from black, thus differing from genes in the orange series. (Figs. 10, 11, 12.)

Two mosaics, each with left eye strikingly banded, were produced by mothers heterozygous for ivory and for cantaloup. The normal brothers had eyes of the three expected colors,—black (OC), cantaloup (Oc), and white (ivory, o^iC, or ivory cantaloup, o^ic). Each mosaic had right eye orange or cantaloup in color which might be genetically cantaloup, Oc; ivory, o^iC, modified by the presence of O in the mosaic; or ivory cantaloup, o^ic. The last possibility is very unlikely since orange cantaloup, oc, is almost white and cantaloup with modified ivory, o^ic, should be no darker. The banded left eyes are shown in Figs. 13 (mosaic No. 550) and 14 (mosaic No. 540). Number 550 shows white dorsally bounded by a sharply marked-off narrow horizontal black band which is split anteriorly by an orange region into which it grades imperceptibly. A broad horizontal white band then follows, sharply marked off from the black lying dorsally, and likewise from a narrow horizontal black line below, which grades ventrally through orange to ivory. Number 540 shows a somewhat similar pattern (Fig. 14), but the median light band is distinctly pink, "cantaloup." It seems most reasonable to suppose that the sharply marked-off light regions of these eyes are genetically cantaloup, Oc, whether they appear white or pink, since eyes of cantaloup stock vary from white to red, becoming progressively darker with age. The grading ivory or orange together with the black bounding bands are then ivory, o^iC, modified by the dominant allelomorph, O, in the cantaloup, Oc, regions. It is interesting to note that black color develops despite the absence of wild type, OC, tissue. Ocelli of No. 550 were colorless, which may have been either cantaloup or ivory, probably the former. In the case of No. 540, ocelli (Fig. 15) are mixed, the right ocellus as well as the right halves of the median and the left being colorless, "cantaloup." Brown pigment in the left halves of the median and left ocelli indicates modified ivory as in the left compound eye. Asymmetry in ocellar size and in pigmentation of ocellar region is to be ascribed to genetic difference in the tissues involved (Whiting, P. W., 1932).

Females heterozygous for stumpy, *st* (legs) (Chromosome VI), produced fourteen males mosaic for stumpy and three that were modified stumpy and thus suspected of being mosaics. Modifications of stumpy will be discussed below.

Males mosaic for various other traits the genes for which have not yet been shown to be linked have been produced by heterozygous mothers. These include two for semilong, *sl*, showing in antennæ, wings, legs; three for tapering, *ta*, showing in antennæ; one for yellow, *Y* (antennæ); twenty-one for shot vein, *sv* (wing veins); three for club, *cl* (tarsi); three for cut, *ct* (wings); one for indented, *in* (wings); one for attenuated, *at* (antennæ), one for twisted, *tw*, showing in antennæ. Certain males from heterozygous mothers have been suspected of being mosaic for other genes but traits are too fluctuating to assert this with certainty.

Origin of Traits in Gynandromorphs

Most of the gynandromorphs found in *Habrobracon* are from pure stock or among the progeny of recessive females crossed with dominant males. The reason for this is that they come only from fertilized eggs and that very few offspring are bred from mated heterozygous females or from dominant females by recessive males. By far the greatest number of mated females that have been set are recessive and are used in connection with studies of ratios of biparental males.

Thirty gynandromorphs have been found among progeny of orange-eyed defective, *d* (r_4 wing vein) females crossed with type males. While sex of antennæ can be readily determined, compound eyes vary considerably in size so that there is no consistent sex difference. Color of eyes is, however, correlated with sex of antennæ. Among the fourteen with both antennæ male, eleven had orange eyes while three had eyes with some mixture of black and orange. Among the fifteen with one antenna male, the other female, eleven had eyes asymmetrical in color with the eye on the female side black, in which case the eye on the male side was orange or mixed; or with the eye on the female side mixed, in which case the eye on the male side was orange. Of the other four cases with asymmetrical antennæ, two had both eyes mixed while two had both eyes black. One wasp with both antennæ female had both eyes black. Male regions of eyes then are orange, matroclinous; female regions show dominant black, patroclinous trait, and are presumably biparental.

Ocelli are larger in male than in female and frequently in gynandromorphs there is asymmetry, the female side showing the smaller ocellus surrounded by integument with characteristically less pigment. Among twenty-seven of these gynandromorphs sixteen had large male ocelli, orange in color, while five had small female ocelli. Six had ocelli asymmetrical in size and color, larger (male) and lighter on one side. In three cases the male ocelli were typically orange while in the other three they had more or less brown pigment. In five cases the female ocelli were described as "black" while in the other one the color was "brown."

Figures 17, 18, and 19 illustrate distribution of pigment in mixed eyes of gynandromorphs from orange females crossed with black-eyed males. Number 373 (Fig. 17) had small (female) black ocelli, and female antennæ. It may be noted that the right eye is black (female) anteriorly. The left eye is orange (male). Number 375 (Fig. 19) is somewhat the reverse with male antennæ, large (male) orange ocelli, while the left eye is orange (male) anteriorly. The right eye is orange (male). Number 397 (Fig. 18) shows a banded condition in the left eye with orange male ocelli, male antennæ, and orange region anteriorly in the right eye. The grading margin may be noted between black and orange in these gynandromorphs in contrast to the clear-cut boundary between black and cantaloup or black and white.

Wings of males are smaller than wings of females. The gene for defective r_4 vein, d, permits fluctuation in the character. Grade 4 denotes the complete absence of the vein from a wing. Heterozygous females frequently show breaks classified as grades 1 or 2. Among the fourteen gynandromorphs with asymmetrical wings, the larger wings (female) had r_4 classified as follows: type 10, d1—3, d2—1, while the smaller wings (male) had r_4 classified: d2—2, d3—5, d4—7. In each individual the male wing showed the greater defect indicating maternal origin.

We have already seen that mosaic males produced by mothers heterozygous for ivory usually show modification of this ivory color to orange. One gynandromorph (No. 322) from ivory female by black male had female abdomen and male head. Compound eyes were ivory showing no modification although female parts of body presumably were Oo^i. Ocelli were male with a trace of brown pigment. Another (No. 481) from a similar cross with head male, thorax and abdomen mixed, likewise had ivory eyes but in this case the ocelli (male) contained red pigment. Another (No. 605) from a female heterozygous for ivory crossed with a black male had male head, female abdomen. Eyes were pale orange, but ocelli (male) were colorless. Another (No. 304) from ivory female by black male had head mixed, abdomen female. Left antenna was female, right male. Eyes were pronounced orange dorsally, black ventrally. Ocelli were male and of light color but showing some brown pigment. Another (No. 296) from a female heterozygous for ivory by an orange male, had one antenna male, the other female. Eyes were ivory, ocelli male and colorless. Other gynandromorphs from crosses of females recessive for various traits by dominant males have shown male structures recessive (matroclinous) in the following cases; two for orange eyes, *o*; three for cantaloup eyes, *c*; three for long antennæ, *l*; one for type recessive to Minnesota yellow antennæ, *My*. Gynandromorphs from similar crosses have shown female structures, ocelli, dominant (patroclinous, presumably biparental), black in five cases of orange and in two cases of cantaloup.

Only four cases have thus far been reported indicating that maternal contribution to male and to female parts is different. These are cases of crosses of heterozygous females by recessive males. One involved reduced, *r* (wings), showing one reduced and one type primary. Three involving orange had black and orange regions in the eyes. Ocelli were orange and female in one (No. 513) so we may suppose the black parts of the eyes were male, the orange female. In the second (No. 602) the larger ocellus (male) was orange, the smaller two (female) were black. In the third (No. 526) the smaller (female) lateral ocellus had no pigment. It was in a darker (male) region. The median and left ocelli contained orange pigment and although large (male) were in a somewhat lighter (female) region.

MODIFICATION BY MOSAICISM OF FUSED AND STUMPY LEGS

The mosaic "mutant" to fused (No. 507) mentioned above had antennæ typical for fused. The left was slightly longer than the right.

Left wings were both type; right were both fused, the primary showing characteristic indentation at tip of radius vein, the secondary shorter than its mate on the left. The three left legs were type; the right showed tarsi with segments typically fused.

The propleuron was darker on the right side which was presumably composed of tissue bearing *f*. Since this specimen came from pure inbred stock it is likely that this pigmental difference is due to the factor *f* itself, in other words *f* is one of a number of genes causing darkening of integumental pigment. This mosaic proved sterile in observed matings with three females.

One of the mosaic males (No. 510) from a female heterozygous for fused had left antenna presumably type, but terminal segments were somewhat fused. Right antenna was fused and rather shorter than the average for this trait (Figs. 22 and 23). Primary wings were type but right secondary was short, probably fused. Left legs were type as was also the third right leg. First and second right legs were fused, the former showing much swelling in femur and tibia as is often the case in fused. Tarsus of this leg had joints completely fused (Fig. 24). Fusion of joints on the second right tarsus was incomplete (Fig. 25) so that it is possible to distinguish the five segments. The mosaic was highly fertile. Tests showed that it bred as wild type.

The other mosaic for fused (No. 490) was produced by a female which was also heterozygous for semilong, *sl* (antennæ, wings, and legs). The wings of this specimen indicate that the two types of tissue present were wild type and semilong fused, for the left wings were both normal while the right showed the combined influence of semilong and fused. Eyes and ocelli were all cantaloup, but this gene does not presumably affect legs or antennæ. Abdomen was normal male but external genitalia were missing (deficiency) except for a small clasper on the right.

Left antenna was type but showing fusion of joints terminally. Right antenna was fused but rather long, perhaps due to the presence of the gene *sl* (Figs. 28 and 29).

The prothoracic right tarsus was type, while all the others were fused but showing more or less segmentation (Figs. 30–34). The metathoracic right was essentially similar to the left. A metathoracic tarsus from stock fused is shown for comparison (Fig. 35).

It is evident that these three mosaics illustrate considerable modification in the fused tarsi. No such difference occurs in fused stock or in semilong fused. Presumably the change in the legs at least is due to mosaicism. Whether the modification of the type (?) antennæ is caused by mosaicism cannot be established from the two specimens.

Deficiency in genitalia of No. 490 may suggest correlated deficiency in antennæ (Whiting, P. W., 1926).

Distribution of type legs as associated with degree of modification of fused may be noted. In No. 507 there is no modification; all the type are on one side, all the fused on the other. In No. 510 all are type on the left. On the right the third leg is type, the middle tarsus much modified fused, while the first is unmodified fused. In No. 490 the five fused tarsi show modification but the middle right which is next to the type first leg is extremely modified in contrast to its mate on the left. The left mate of the type leg shows definite but relatively little modification. These relationships suggest that modification of fused may be greater according to proximity to type on the same side but that there is little if any influence across the median plane. It may be noted that in No. 490 in which there are no type legs on the left, the left antenna is type and the degree of modification is less extreme on this side. Little of significance may be based on these three specimens, but in view of the relationships in modification of stumpy, it is regarded as worth while to call attention to these facts.

In stumpy wasps the tarsal claws are very close to the tibia, the tarsal segments being reduced to minute chitinous vestiges (as in Fig. 39). Examination of over seventy-five specimens showed only six with a small tarsal segment on one or more legs. Legs showing this segment were prothoracic three, mesothoracic ten, and metathoracic one. There is therefore greater tendency for mesothoracic to possess a segment than for the other pairs. Moreover, an individual having a tarsal segment on one leg is likely to have it on others, for four of the six had segments on two legs while two had segments on three legs.

Females heterozygous for stumpy have been bred in X-radiation experiments by Raymond J. Greb. Among the progeny there have been produced fourteen males that were mosaic for stumpy and three which, although not possessing any type legs, showed modification of stumpy and were mosaic for other traits. Modified stumpy legs of mosaics are shown in Figs. 36, 37, and 38.

Of the entire group of seventeen, ten were produced by females X-rayed as adults, while seven were from non-rayed material. The modification of stumpy found in mosaics appears to have no relation to X-radiation of the mother.

Among the fourteen mosaics for stumpy there were thirty-six type legs and forty-eight stumpy. There were only two instances of right and left pairs of type legs but nine of the fourteen mosaics had all type legs on one side, all stumpy on the other. This distribution of type and stumpy tissue is considered due to the distribution of genetically diverse nuclei in cleavage.

In order to have a quantitative measure of modification of stumpy, the legs of the seventeen mosaics (fourteen mosaic for stumpy) were roughly classified in grades from 0, denoting typical stumpy, to 6 with tarsi considerably over half normal length. The legs of the three mosaics that were not obviously mosaic for stumpy (with no type legs) were grade 0—10, grade 1—3, grade 2—4, grade 4—1.

Stumpy legs that were opposite to type legs were grade 0—23, grade 1—3, grade 2—3, grade 4—3. Stumpy legs that were on the same side as type legs were grade 0—2, grade 2—1, grade 4—7, grade 6—2. It therefore appears that modification of stumpy is due to association with type on the same side and that there is very little if any influence across the median plane.

If the grades of the various stumpy legs are totalled, the twenty prothoracic amount to 19; the twenty-four mesothoracic amount to 45; and the twenty-two metathoracic amount to 12. It therefore appears that mesothoracic are most subject to modification, prothoracic less, and metathoracic least. This order agrees with the six cases found in non-mosaics, but both ratio of legs modified and degree of modification is much higher in mosaics than in non-mosaics.

A Haplo-diploid Male Mosaic

A certain type of male mosaic not previously considered is a logical expectation from the fact that biparental (diploid) males appear in certain crosses and from the theory that a gynandromorph may be produced when one nucleus of a binucleate egg is fertilized.

Shortly after the discovery of the mutation orange, there were found five patroclinous males from orange females crossed with black males, which, although showing no obvious mosaicism, bred as orange and were highly fertile (Whiting, P. W., 1921). The hypothesis was at that time advanced that the " black " parts were haploid and strictly androgenetic. In view of the abundant evidence now accumulated for egg binuclearity and for male diploidism as well as lack of evidence for androgenesis, it seems preferable to regard these patroclinous male mosaics as haplo-diplonts, developing from binucleate eggs in which one nucleus was fertilized and gave rise, not to female parts as in a gynandromorph, but to diploid male parts.

Such a male, actually showing mosaicism, has recently been found and tested by Milton Franklin Stancati. An orange female (Stock 3) was crossed with a black-eyed male. This male was the original mutant to indented, *in* (wings). In addition to the regular black females—eighteen, and the orange males—nine, there were produced seven black-eyed biparental males, two of which occurred in the same vial (d) with the mosaic (No. 544).

The mosaic was entirely male, with twenty-four segments in each antenna. Eyes were orange, matroclinous, except for black posterior region of the left. Ocelli were symmetrical in size and large (male). There was much brown pigment in left and median. The right had a very small amount of brown pigment (modified orange?). Body pigment was symmetrical.

The mosaic was highly fertile, producing seventy daughters when mated with three females which produced forty-three sons (female ratio 62 per cent). By the two orange mates there were produced fifty-four orange daughters, while by a black-eyed heterozygous sister there were produced ten black and six orange daughters including no indented although three indented males appeared and one non-indented, proving this sister heterozygous as expected. There were also no indented among numerous descendants of the mosaic by the Stock 3 females. The mosaic therefore bred like its maternal stock (No. 3).

Since eight of the twenty-six fertilized eggs developing in the fraternity of No. 544 produced males, a binucleate egg would here have a very good chance of producing a haplo-diploid male mosaic. In view of the fact that eye color alone has generally been used as the criterion of male biparentalism, it is highly probable that haplo-diploid male mosaics have been missed on account of failure of obvious mosaicism, being classified either as normal haplonts or as biparental males. On the theory advanced, their frequency may bear the same relation to gynandromorphs as the frequency of biparental males bears to females, —haplo-diploid male mosaics/gynandromorphs = biparental males/females.

A Case of Four-strand Crossing-over

Mosaic males from binucleate eggs would be expected to possess in their two types of tissue two of the various possible combinations of those genes for which their mothers were heterozygous. Only in those cases, however, in which the two or more pairs of genes affect the same structure can we be sure of what combinations are present. Thus, if eyes are mosaic for type (black) and cantaloup and wings are mosaic for type and long, there is no way of telling which wing is genetically cantaloup or which eye is genetically long. Virgin females heterozygous for long and for reduced have produced mosaic males having wings long on one side, reduced on the other, or having wings wild type on one side, long reduced on the other. Gynandromorphs have likewise come from reciprocal crosses of orange and cantaloup having eyes type (black) if female, or showing the maternal color if male.

One instance of recombination of genes affecting the wings may be noted as it involves four-strand crossing-over in ovogenesis. In connection with her experiment on the production of impaternate females, Kathryn A. Gilmore bred progeny from a virgin female heterozygous for four factors in Chromosome II, *My/c.l.n.* Minnesota-yellow, *My*, an irregular dominant, affects basal segments of antennæ. It lies well to the left, 20 units ±, of the factor for cantaloup eyes, *c*. Long, *l*, lying 12 units ± to the right of cantaloup, shortens wings distally but lengthens antennal segments. Narrow, *n*, about three units to right of long, makes wings narrow. Among the progeny (male) segregating these four factors as expected there occurred a male (No. 552) with Minnesota-yellow long antennæ and cantaloup eyes and ocelli. Left primary wing was narrow (Fig. 26); right, long narrow (Fig. 27) with defective, r_4, venation. Character of antennæ indicates a crossover somewhere between *My* and *l*. Origin of defective is uncertain and need not concern us. Left primary wing indicates that a crossover occurred between *l* and *n*. The right primary, however, shows a noncrossover combination.

The facts indicate that we are concerned with a two-crossover oöcyte, two strands crossing over between *My* and *c*, the other two between *l* and *n*. There is no evidence for double crossing-over. Crossing-over between two strands in one region does not then prevent crossing-over between the other two in another region. For *My* and *n* we have prereduction; for *c* and *l* post-reduction. The two oötid nuclei involved in parthenogenetic cleavage were *My.c.l.n* and *My.C.L.n,* while *my.my.Cc.Ll.Nn* went out in the first polar body.

SUMMARY

Mosaic males from heterozygous mothers have shown clear-cut mosaicism for the recessive eye colors cantaloup and white. Orange, however, shows intergradation with black of wild type and ivory shows complicated types of modification and intergradation. A similar condition obtains in the case of gynandromorphs. Males have shown clear-cut mosaicism for sixteen other traits. As regards either fused or stumpy legs, however, there is much modification, with evidence that influence is from wild type tissue on the same side of the body, but not on the opposite side. Further evidence is presented indicating that gynandromorphs show maternal traits in male parts of body while female parts are biparental. Maternal contribution to male and female parts may be different. A male mosaic has been found which is best explained as a haplo-diplont, being in part biparental. A mosaic male from a mother heterozygous for four linked genes indicates such a combination of traits that two crossovers must have taken place in the tetrad, one between two strands, one between the other two, in the egg from which this male developed.

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Explanation of Plate I

Figs. 1, 2, 3, 5, 7, 9, 10, 11, 12, 13, 14, 17, 18, 19. × 27. In compound eyes solid black represents black, stippling indicates reddish or orange color, white unstippled regions are white.

Fig. 16. × 17.

Figs. 4, 6, 8, 15, 20, 21. × 64.

Figs. 22, 23, 24, 25, 28–39. × 27.

Figs. 26, 27. × 11.

Figs. 1–15 show eyes and ocelli of males mosaic for various traits as follows: Fig. 1. Type and ivory (No. 383). Fig. 2. Orange and ivory (No. 341). Figs. 3 and 4. Type and cantaloup (No. 378). Figs. 5 and 6. Type and cantaloup (No. 465). Figs. 7 and 8. Type and cantaloup (No. 351). Fig. 9. Type and cantaloup (No. 593). Figs. 10 and 11. Type and white (No. 601). Fig. 12. Type and white (No. 600). Fig. 13. Ivory and cantaloup (No. 550). Figs. 14 and 15. Ivory and cantaloup (No. 540).

Fig. 16. Mesonotum of male mosaic for broad, *br* (No. 465).

Figs. 17 (No. 373), 18 (No. 397), 19 (No. 375). Heads of gynandromorphs with male regions of eyes orange, female regions black.

Figs. 20, 21. Left and right eyes, respectively, of male mosaic for glass and for orange (No. 549). Stippling indicates brownish pigment. The glass regions are in general orange.

Figs. 22 (left antenna), 23 (right antenna), 24 (right prothoracic leg), 25 (right mesothoracic tarsus) from male mosaic for fused (No. 510).

Figs. 26 (left, narrow) and 27 (right, long narrow) wings of mosaic male (No. 552).

Figs. 28 (left antenna), 29 (right antenna), 30 (left prothoracic tarsus), 31 (right prothoracic tarsus), 32 (left mesothoracic tarsus), 33 (right mesothoracic tarsus), 34 (left metathoracic tarsus) from male mosaic for fused (No. 490).

Fig. 35. Metathoracic tarsus of typical fused male.

Fig. 36. Left mesothoracic tarsus of male mosaic for stumpy (No. 495).

Figs. 37, 38, 39. Two views of left mesothoracic tarsus and one of right metathoracic tarsus of male mosaic for stumpy (No. 497).

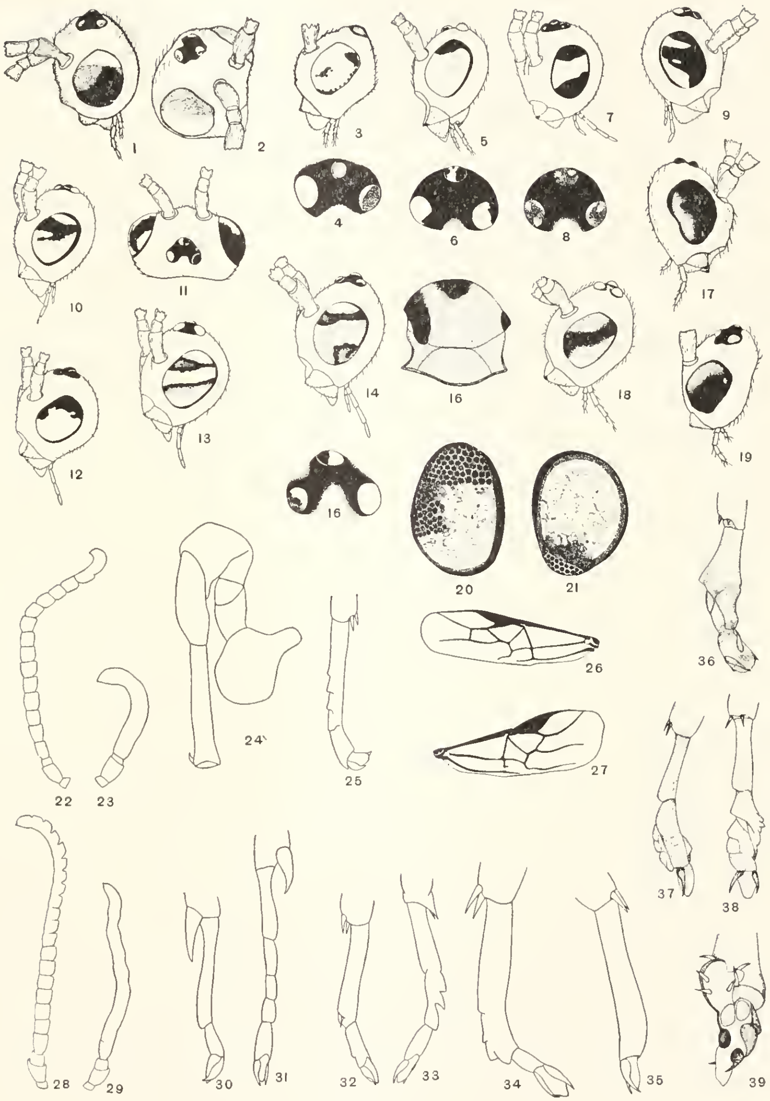

Plate I

TEMPERATURE AND LIGHT AS FACTORS INFLUENCING THE RATE OF SWIMMING OF LARVÆ OF THE MUSSEL CRAB, PINNOTHERES MACULATUS SAY

JOHN H. WELSH

(*From the Zoölogical Laboratory, Harvard University, and the Woods Hole Oceanographic Institution*)

Significant and numerous studies have been made in the past on the phototropism of plants and animals, and the quantitative aspects of the effect of light on photosensory systems have been extensively studied by Hecht and others; however, little is known concerning photokinesis and the effect of temperature on free-moving, light-sensitive organisms. Some investigators have even denied an effect of light on the behavior of such forms other than on orientation. Davenport and Cannon (1897) found an apparent difference in rate of swimming of *Daphnia* in full light as compared with swimming under one-fourth this intensity, but they concluded that this was probably due not to a change in velocity but to more rapid and accurate orientation at the higher intensity. Yerkes (1900) substantiated the findings of Davenport and Cannon on *Daphnia* and found in addition a slight effect of intensity on rate of swimming of *Cypris,* but he came to similar conclusions, namely, that the apparent change in rate was due primarily to changes in accuracy and rapidity of orientation. In neither of these investigations was a very wide range of intensities used. Their conclusions agree with those of the majority of observers before and since, with few exceptions. Moore and Cole (1921) found that the rate of locomotion of *Popillia japonica* during upward geotropic progression was influenced by light and that the rate of movement was a function of the light intensity. Cole (1922*a*) obtained similar results regarding the upward creeping of *Drosophila,* and (1922*b*) found a distinct effect of light on the rate of creeping of *Limulus.* Mast (1923) expresses doubt regarding the validity of Moore and Cole's (1921) results on *Popillia* for he states that they did not exclude the time required for the insects to orient and get under way. Mast also doubts the value of Loeb's (1890) observations on rate of movement of aphids as a function of light intensity, for he claims that temperature was not eliminated as a factor. Mast and Gover (1922) studied the effect of intensity of light and rate of locomotion of *Phacus* and *Euglena,* and although with

Euglena they found what may be a significant increase in rate at high intensities, they nevertheless concluded that light intensities sufficient to cause rapid and accurate orientation need not have any additional appreciable effect on rate.

The lack of agreement regarding the photokinetic effect of light, and the fact that the problem has considerable bearing on our proper understanding of movements of plankton organisms, led to an attempt to gain additional information. Crustacean larvae form one of the representative groups of the animal plankton of the sea and were found to be most satisfactory for the work as planned. The work was carried out during the summer of 1931 at the Woods Hole Oceanographic Institution. It was made possible largely through the kindness of Dr. H. B. Bigelow, Director of the Institution, and the author wishes to acknowledge his appreciation for the excellent facilities and equipment placed at his disposal. The author is also indebted to Professor W. J. Crozier for suggestions in the preparation of the paper.

Material and Methods

For the study of photokinesis a suitable animal should possess certain characteristics which have been lacking in part in many of the animals previously studied. They should be positive or negative to light; they should orient accurately and rapidly; they should move in a straight line; they should preferably be aquatic organisms, in order to make it possible easily to control the temperature. In addition to these requirements any satisfactory experimental animal must be obtainable in large numbers over a considerable period of time, or must be easily reared or kept in the laboratory.

For satisfying these requirements it is difficult to conceive of a more suitable form than the young larvæ of *Pinnotheres maculatus* Say, the mussel crab. The adults are found living as parasites in the mantle cavity of *Mytilus edulis,* the edible mussel. Sixty-five per cent of the mussels collected from a bed near Grassy Island, Woods Hole, were infested with these crabs so the adults were easily obtained. A considerable number of females carrying eggs were found at all times during July and August. These are easily kept in the laboratory in bowls of sea water, and one can have one or more batches of larvæ hatching daily, each batch containing several hundred individuals.

The larvæ are distinctly positive to light. They orient with head away from the light and by means of forward strokes of the swimming appendages move backward toward the light. This is similar to the orientation of the young larvæ of the lobster as observed by Hadley

(1908), and of the young larvæ of *Palaemonetes* as described by Lyon (1906). At all light intensities used there was extremely rapid orientation consuming only a fraction of a second, and the larvæ took a course toward the light which was a straight line in most cases.

At a given light intensity and temperature the velocity of movement was quite constant for individuals of a given age, but after two or three days the rate of swimming decreased and at the age of four to five days the larvæ had a tendency to be temporarily negative; this necessitated using larvæ of known age.

As a careful control of either temperature or light intensity is necessary when studying the effect of the other factor, all experiments were performed in a dark room; by means of a water-bath, the temperature could be accurately controlled, and with proper methods light of the desired intensity obtained.

The water-bath consisted of a 30-gallon insulated tank with 4 x 6 inch plate glass windows set in opposite sides and near one end. Temperatures below room temperature were obtained by means of a cooling unit similar to one described by Stier (1931), with a mercury thermoregulator operating a heating unit of 100-watt capacity. Rate as a function of light intensity was studied for the most part at temperatures slightly above room temperature which obviated the necessity of using the cooling device. The water in the tank was stirred by means of a motor-driven agitator, and was changed at frequent intervals in order to avoid loss of light by suspended particles which tended to accumulate in the tank.

The light-source was a 6-volt, 18-ampere, ribbon filament lamp, shielded by means of a double housing in order to prevent leakage of light. The light passed through lenses which kept the rays practically parallel. The intensity was controlled by means of Wratten neutral tint filters, which, used singly or in combination, transmitted the following percentages of the original light: 50, 25, 10, 5, 2.5, 1, 0.5, 0.1, 0.05 per cent. The beam of light passed through a series of screens with apertures of the proper size, and through ground glass for diffusion. A second light for attracting the larvæ to the opposite end of the trough consisted of a Spencer lamp with a 150-watt bulb and ground glass.

The larvæ were placed in filtered sea water in a trough of plate glass with inside dimensions 29 x 4 x 4 cm. This trough was covered with a glass plate and submerged to within a half centimeter of the top in the water of the bath. Here it was supported on hangers so that it was always at a given distance from the light source and so that the beam of light just covered the inside section of the trough and, the rays being parallel, reflection of light from the glass sides was negligible.

Measurements of the light intensity within the trough were made by means of a Macbeth Illuminometer, by putting a small test plate in the water of the trough. The intensity of light at the end of the trough nearest the ribbon filament lamp (the variable source) when no filters were used was found to be 93 meter candles. The 150-watt lamp at the distance used gave an intensity of 68 meter candles at the end nearest this lamp.

By means of the neutral filters it was possible, without changing the distance of the lamp from the tank, to obtain the following intensities of light at the end of the trough nearest the ribbon filament lamp: 46.5, 23.3, 9.3, 4.7, 2.3, 0.93, 0.47, 0.093, and 0.047 meter candles. The last intensity was the lowest that it was practical to use, for below this it was impossible to see the larvæ distinctly and even at this intensity it was necessary for the observer to be adapted to complete darkness before making each observation. There was, of course, considerable absorption of light by the sea water of the trough, and the intensity at the far end in each instance was considerably lower than the intensity given for the near end, and this varied throughout the trough; however, the total light reaching the larvæ as they swam from the far end to the near end of the trough was proportional to the intensity at the near end, and varied as this was varied.

In preliminary experiments observations were made on the rate of swimming of individual larvæ as compared with the rates of the first or middle member of a small swarm, and the variations were no greater in the second instance than in the first. As it was more often possible to complete a series of data if several individuals were used instead of one, a small swarm of 10–25 animals was used in most of the experiments. The writer realizes the possibility of greater variations in the results when swarms are studied but in this particular instance the behavior of several larvæ selected from a given batch showed as great uniformity as did single individuals, at least when the fastest and slowest members had been discarded.

In a particular experiment a group of larvæ were selected of the proper age and placed in the trough and adapted to the temperature of the water-bath for at least a half hour. They were then attracted by the 150-watt light to the end of the trough away from the variable light source, and their swimming movements would keep them in close contact with the glass in their endeavors to continue their course toward this light. This light would then be cut off and at the same time the light at the opposite end turned on and a stop watch started. The time necessary for the fastest individual or group of individuals to traverse the 29 cm. was then taken. At a given light intensity or temperature either five or ten trials were made and the results averaged. In this way it was possible to determine the rate of swimming of the larvæ both as a function of temperature and light intensity. As stated above the extremely rapid orientation of the larvæ and the apparent absence of a latent period obviated the necessity of considering these factors and made the measurements easier and more accurate than would have been true with many free-swimming forms.

TEMPERATURE AND VELOCITY OF LARVÆ AT A CONSTANT LIGHT INTENSITY

Preliminary observations indicated a decided effect of temperature on the rate of swimming of the larvæ, and although only one series of measurements on individuals of the same age was made over a wide range of temperatures, the results from this were quite significant. The range of temperatures used was from 27.0° C. to 13.4° C. The light toward which the larvæ swam was kept at a constant intensity of 93 meter candles. The results are shown in Table I. The times given for swimming 29 cm. are, at each temperature, averages for ten readings. Between different temperatures one hour was allowed for adaptation to the new temperature.

TABLE I

Effect of temperature on rate of swimming of larvæ at a constant light intensity

Temperature	Time for swimming 29 cm. (Averages of 10 readings)	P.E. of time	Velocity $\frac{1}{\text{time for 29 cm.}}$
° C.	*seconds*		
27.0	12.38	± 0.100	0.0806
25.9	13.09	± 0.090	0.0763
24.8	14.53	± 0.046	0.0690
23.5	15.50	± 0.057	0.0645
22.0	17.70	± 0.099	0.0565
20.0	20.70	± 0.127	0.0483
18.5	24.79	± 0.175	0.0403
17.6	25.64	± 0.117	0.0391
16.5	27.72	± 0.139	0.0361
15.2	32.30	± 0.152	0.0310
14.2	36.11	± 0.149	0.0277
13.4	39.05	± 0.302	0.0256

From these results one can determine the time for swimming a meter at different temperatures. At 27.0° this was 43.4 seconds, under the conditions of the experiment; at 18.5° the same larvæ required 1 minute 27 seconds for travelling a meter; and at 13.4°, 2 minutes 16.5 seconds.

Temperature changes in the sea are slow and comparatively small yet this factor must be of some importance in determining the rate of movement of plankton organisms, particularly when near the surface.

The probable errors of the times are shown in Table I and it may be seen that these are not large. There is a rather definite relationship between the average time for swimming 29 cm. and its probable error, and it is interesting to note that they are both affected by temperature in much the same way; as the mean time increases, the P.E. increases in proportion. The significance of this has been pointed out by Crozier (1929) and Navez (1930).

A plot of velocity against temperature centigrade (Fig. 1) indicates that the increase in velocity with increasing temperature is not on a simple smooth curve; it is evident that one curve does not fit the results. There is a break near 18.5° and near this point occurs the one velocity measurement which does not conform fairly well with the rest. It will be seen in Table I that the mean time from which this velocity was obtained has a high P.E. compared with those above and below. When the logarithm of the velocity is plotted against the reciprocal of the absolute temperature (Fig. 2) it is more apparent that between 13.4° and 27.0° two lines must be drawn to fit the data; these lines are straight and intersect at about 18.5°, thus indicating that the Arrhenius equation $\left[\frac{K_2}{K_1} = \epsilon^{\frac{\mu}{2}\left(\frac{1}{T}-\frac{1}{T_2}\right)}\right.$ where K_1 is the velocity at T_1° Abs., and K_2 the velocity at T_2° Abs.; $\epsilon = 2.718$; μ is a constant over a certain tempera-

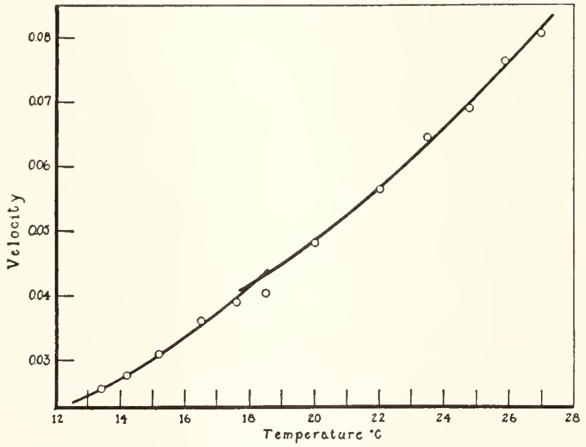

FIG. 1. Data of Table I plotted as velocity (reciprocal of time for 29 cm.) against temperature centigrade. Two curves are shown which intersect between 18° and 19°. This break is more evident when the same data is plotted as in Fig. 2.

ture range and designated by Crozier as a "temperature characteristic"] holds for the rate of swimming of these larvæ as a function of temperature. The values of μ as calculated are 16,900 below 18.5° and 12,800 above 18.5°. It is not necessary to go into the significance of these values and of the break, or critical temperature, for this has been done by Crozier (1924) and others, for many cases which are fundamentally similar; it is sufficient to note that corresponding values for μ have been frequently encountered in studies of the influence of temperature on rate of many biological processes.

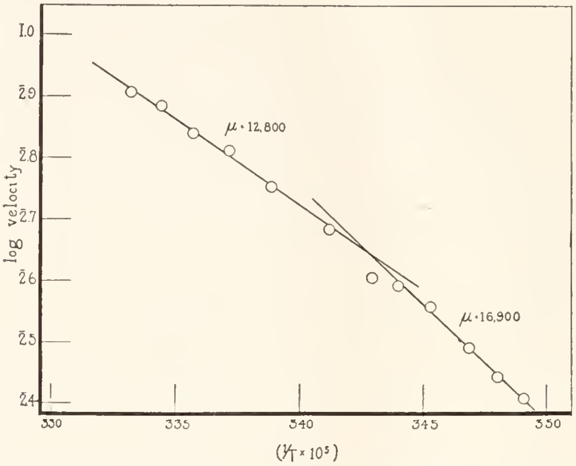

FIG. 2. Same data as in Fig. 1, plotted as the logarithm of velocity against the reciprocal of absolute temperature × 10^5. That two lines must be drawn to fit the data is evident.

Few studies of the effect of temperature on the rate of locomotion of organisms have been made, the only other on a free-swimming form with which the writer is familiar is that of Glaser (1924) for *Paramecium*. He found that the Arrhenius equation could be applied, and secured a value for μ of 16,000 below 16° and of 8,000 above 16°.

It is realized that changes in density and viscosity of the sea water occur with changes in temperature and these factors enter in, and introduce slight errors in the measurements which are difficult to eliminate. To what extent they are significant remains to be seen.

The effect of temperature on the velocity of swimming at different light intensities will be discussed in a later section.

LIGHT INTENSITY AND THE RATE OF SWIMMING OF LARVÆ AT A CONSTANT TEMPERATURE

The importance of temperature as a factor influencing the rate of swimming of *Pinnotheres* larvæ has been indicated in the previous section. During the work on the photokinetic effect of light the temperature was carefully controlled and kept constant, for a given series, within ± 0.1°.

In addition to temperature, age was found to be an important factor influencing the rate of locomotion, and although this was not carefully investigated the age was taken into consideration in the later determinations of the effect of intensity of light. Because either age or temperature varied between separate series of experiments in some of the earlier work, it was impossible to average or compare much of the data, although the results were fundamentally similar. As an example of the effect of age on rate of movement it is necessary to cite only one instance. On August 23 at 2:00 P.M. a certain larva swam the 29 cm. in 14.2 seconds, in a light intensity of 93 meter candles; on August 24 at 10:00 A.M. the same individual required 15.2 seconds to swim the same distance. In each case the times as given are averages for five readings. It was also found that over a range of intensities, larvæ 30 hours old were less sensitive to the light than larvæ 15 hours old. It is obvious that age would not be of such importance if one were dealing with adult animals, but in the case of crustacean larvæ, as is well known, a few days makes a great difference in the responses of the animals to light (Hadley, 1908, and others).

Early in the course of the work on light intensity it was found that changes in rate of swimming were obtained over a comparatively small range of intensities. The maximum velocity, at temperatures near 23°, was reached at approximately 25 meter candles. This suggests that in much of the previous work on the photokinetic effect of light, intensities above the minimum necessary for eliciting a maximum response may have been used, and the conclusions that light intensity has no effect on rate of locomotion are perhaps unjustified in many instances. It is logical to assume that aquatic organisms are sensitive to a lower range of intensities of light than are land organisms, for they live constantly at reduced intensities.

It should also be pointed out that the intensity of light to which the animals are previously adapted affects to a certain extent the rate of movement in subsequent intensities. Several trials were made to determine the effect of dark adaptation on rate of movement in the light, with the expectation that for a few trials the animals would swim more rapidly, due perhaps to an accumulation of a photosensitive material in the light receptors. In every instance the first few trips, after dark adaptation of several hours, occupied more time than subsequent trips. This phenomenon is perhaps similar to that noted by Davenport and Cannon (1897), who found that *Daphnia* responded more quickly and accurately to the light after having made several trips in it. Hecht (1925) also found in *Ciona* that after dark adaptation of several hours the first reaction time to a given intensity of illumination was definitely longer than those which followed, and which remained constant for long periods. These authors offered no explanation and as yet there seems to be no satisfactory reason why this should be true. The following experiment seems somewhat contradictory in view of these results on dark adaptation. Larvæ were adapted to a series of light intensities ranging from 0.093 to 93.0 meter candles and the time for swimming toward a light of 68 meter candles subsequently obtained. The results are shown in Table II. After adaptation to light of 0.093 meter candles 13.4 seconds were consumed in swimming 29 cm., in an illumination of 68 meter candles. This time increased as the intensity of the adapting light increased, until after adaptation to 93.0 meter candles 14.7 seconds were required for swimming the same distance. This indicates a distinct effect of the adapting illumination, and in the experiments to follow the larvæ were, in every case, adapted to light of a constant intensity before each trial.

TABLE II

Effect of intensity of adapting light on time for swimming 29 cm. toward light of constant intensity. Age of larvæ 30 hours. Temperature 25.4° C.

Intensity of adapting light	Intensity of attracting light	Time for swimming 29 cm. toward light of 68 meter candles
meter candles	*meter candles*	*seconds*
0.093	68	13.4
0.93	68	13.3
4.7	68	13.9
9.3	68	14.2
23.3	68	14.2
46.5	68	14.3
93.0	68	14.7

From several series of experiments to determine the velocity of swimming in intensities ranging from 0.093 or 0.47 meter candles to 93.0 meter candles very uniform results were obtained. The maximum rate of swimming was reached in light of about 25 meter candles, and although this rate varied somewhat the variation was due to differences in age of the larvæ, temperature of the water, or in some instances perhaps to slight differences between given lots of larvæ or individual larvæ.

Table III gives one such series of data on larvæ 20 hours old, at a temperature of 24.5°. In this series readings were begun at the higher intensities and the intensities of the variable light source decreased by definite amounts between sets of readings. Progression from low to high intensities yields essentially the same results. In this particular series the faster individuals in the swarm travelled the 29 cm. in 16.6 seconds, at an intensity of 93.0 meter candles. At 46.5 meter candles there was no significant change. At 23.3 meter candles the time for swimming 29 cm. had increased to 17.5 seconds and from this intensity down to 0.47 meter candles, the lowest intensity tried, there was a gradual increase in time; at the lowest intensity the time being almost exactly twice as great as at the higher intensities.

A plot of this data as reciprocal of the time against the light intensity yields a smooth curve as seen in Fig. 3. One point does not fall well on the curve but, as may be seen in Table III, the probable error of the time at this intensity (9.3 meter candles) is high in comparison with those above and below. The velocity at 93.0 meter candles is not shown on the graph as it is practically the same as that at 46.5 meter candles. It should be noted that in no instance were rates of swimming obtained in very low intensities or in total absence of light, for obvious reasons. It should also be pointed out that swimming movements of crustacean larvæ do not stop even in absence of light; instead the larvæ remain at or near the surface, kept there by constant but random movements.

Table III

Effect of light intensity on rate of swimming of larvæ at a constant temperature (24.5° ± 0.1). Series 8.6. Age of larvæ 20 hours.

Intensity	Time for 29 cm. (Averages of 5 readings)	P.E. of time	Velocity $\frac{1}{\text{time for 29 cm.}}$
meter candles	*seconds*		
0.47	33.9	± 0.91	0.0295
0.93	31.8	± 0.80	0.0315
2.3	27.2	± 0.70	0.0368
4.7	23.5	± 0.57	0.0426
9.3	21.5	± 0.62	0.0465
23.3	17.5	± 0.31	0.0571
46.5	16.7	± 0.41	0.0599
93.0	16.6	± 0.22	0.0602

One might expect, if the effect of light on rate of locomotion could be determined in the same way as the effect on orientation of the larvæ, to find them obeying the Bunsen-Roscoe Law as did Loeb and Northrop (1917) in their investigation of the orientation of *Balanus* larvæ to a two-point source of light. If this law held for velocity of locomotion, the relationship between velocity and light intensity would be a linear one. That this is not true is quite evident, and the reason is perhaps obvious. Loeb and Northrop were concerned with the degree of turning of the path toward the stronger of two lights and this in no way depended on previous velocity and to only a very slight extent on water resistance.

The results obtained (Fig. 3) more nearly resemble those obtained by Moore and Cole (1921) for rate of movement of *Popillia japonica* as related to light intensity, and later by Cole (1922) for *Drosophila*. In both of these instances they found apparent conformity with the Weber-Fechner Rule, as Henri (1912) had claimed for the reactions of *Cyclops* to ultra-violet light, and Patten (1915) when using a graded series of absolute intensities of opposed lights in studying orientation of blowfly larvæ. Although such conclusions are to a certain extent unwarranted by the fact that the Weber-Fechner Rule does not hold for intensity discrimination in certain forms, as pointed out by Hecht (1924, 1928), yet the approximate linear relation of response plotted against the logarithm of intensity is sometimes useful in analyzing such data. Crozier (1928) in discussing the case of *Limax,* where the

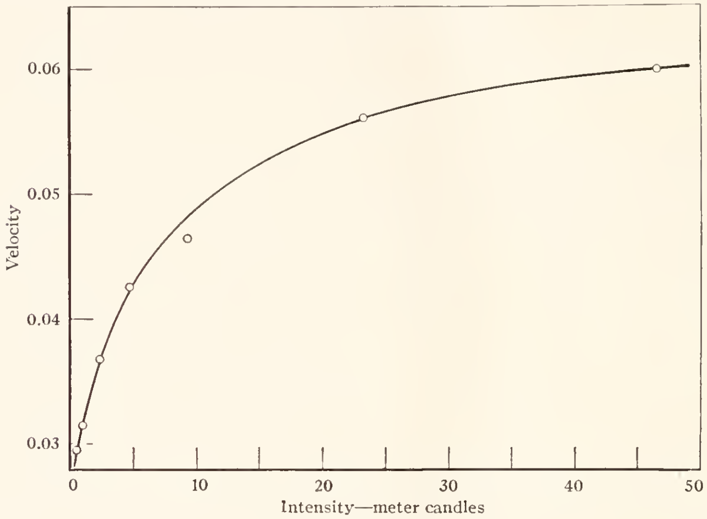

FIG. 3. Data of Table III, Series 8.6, plotted as velocity against intensity (meter candles). See text.

amount of turning per unit length of path is directly proportional to the logarithm of the light intensity, emphasizes the fallacy of considering this an obeyance to Weber's Rule and yet suggests that the same empirical treatment is useful, where other perhaps more significant treatments are impractical.

If the data given in Table III are plotted as velocity against the logarithm of the intensity, the relationship is found to be far from linear. This indicates that the velocity is not related directly to the logarithm of the intensity of illumination. In the work of Moore and Cole on the Japanese beetle they were dealing with an organism which, under a ruby light or in the dark, seldom showed any movement, but which was aroused to activity by illumination from any direction. As has been pointed out above, *Pinnotheres* larvæ are constantly moving even in total absence of light, and this initial velocity must bear some relation to later velocities produced by illumination.

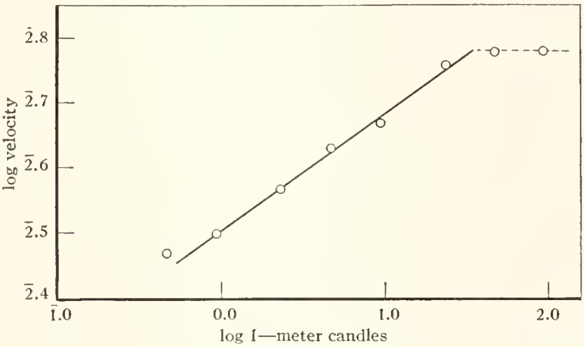

FIG. 4. Complete data of Series 8.6 plotted as logarithm of velocity against logarithm of intensity. The graph is essentially rectilinear until the maximum velocity of swimming is approached.

If we assume that the velocity of movement (V) is so related to the light intensity (I) that any increase in velocity (ΔV), produced by a small increase in intensity (ΔI), is a function of the velocity and also inversely proportional to the intensity, we obtain the following expression:

$$\frac{\Delta V}{\Delta I} \propto \frac{V}{I} \, .$$

Upon integration this yields the following equation:

$$\log V = k \log I - C$$

where k is the constant for the slope of the line, at any given temperature, and C is an integration constant. This expression indicates the way in which the slope of the curve of velocity plotted against intensity depends, at any point, on both the previous velocity and light intensity. It may be checked by altering another variable such as temperature as is shown in the following section. If we apply this formulation to the data in Table III and plot the logarithm of velocity against the logarithm of intensity, we obtain a straight line over most of the range of intensities as shown in Fig. 4. Two velocities are shown at intensities of 46.5 and 93.0 meter candles which were obtained at or near the maximum velocity possible at this temperature, and which, of course, do not fall on the curve as drawn, but between 0.47 and 23.3 meter candles the straight line fit is good.

The theoretical significance of such an empirical treatment need not concern us. It is sufficient that we have a convenient method for comparing data obtained by varying the several factors such as temperature, light intensity, and age, which so evidently influence the rate of swimming.

Table IV

Effect of light intensity on rate of swimming of larvæ at different temperatures. Age of larvæ 15 hours.

INTENSITY	SERIES 8.21 A TEMP. 13.4° ± 0.1°		SERIES 8.21 B TEMP. 18.0° ± 0.1°		SERIES 8.21 C TEMP. 27.1° ± 0.1°	
	Time for 29 cm.	P.E. of time	Time for 29 cm.	P.E. of time	Time for 29 cm.	P.E. of time
meter candles	*seconds*		*seconds*		*seconds*	
0.093					22.8	± 0.28
0.47			39.2	± 0.62	18.3	± 0.28
0.93	86.7	± 2.16	35.9	± 1.04	17.1	± 0.06
2.3	64.7	± 1.06	31.0	± 0.39	15.2	± 0.21
4.7	56.8	± 0.89	27.0	± 0.35	13.7	± 0.09
9.3	47.7	± 0.53	23.9	± 0.32	13.5	± 0.11
23.3	39.7	± 0.30	22.4	± 0.33	13.3	± 0.17

The Effect of Light Intensity on Rate of Swimming of Larvæ at Different Temperatures

In the preceding sections we have shown the effect of temperature on the velocity of *Pinnotheres* larvæ at a constant light intensity, and the effect of light intensity at a constant temperature. Now it might be of value to compare light intensity curves from larvæ of the same age obtained at different temperatures.

Table IV gives three series of data on larvæ 15 hours old, at temperatures of 13.4°, 18.0° and 27.1° C. The complete range of light intensities available was not used at each temperature, for the rate of swimming was extremely slow and rather irregular below an intensity of 0.93 meter candles, at the lowest of the three temperatures; however, sufficient determinations were made for adequate comparisons.

The curves for velocity plotted against light intensity are shown in Fig. 5. It may be seen that with increasing temperature, there is distinct displacement of the curves upward, and an earlier arrival at the maximum rate of swimming as the temperature increases. Also the slope of the curves changes with the temperature. At the lowest temperature the curve is much flatter than at the higher temperatures. While it was impossible to determine the effect of temperature upon the velocity of swimming in absence of light, it will be seen that there is a distinct effect on rate of locomotion in the dark, and if the curves were begun at the zero point on the abscissa, they would intercept the ordinate at varying levels above zero, depending upon the temperature. This is due to changes in rate of general activity, and to changes in the viscosity of the sea water. While no attempt has been made to correct for viscosity changes due to changes in temperature, these are of considerable importance. It was shown by Ostwald (1903, *a* and *b*) that in comparison with water, which might be considered to have a viscosity

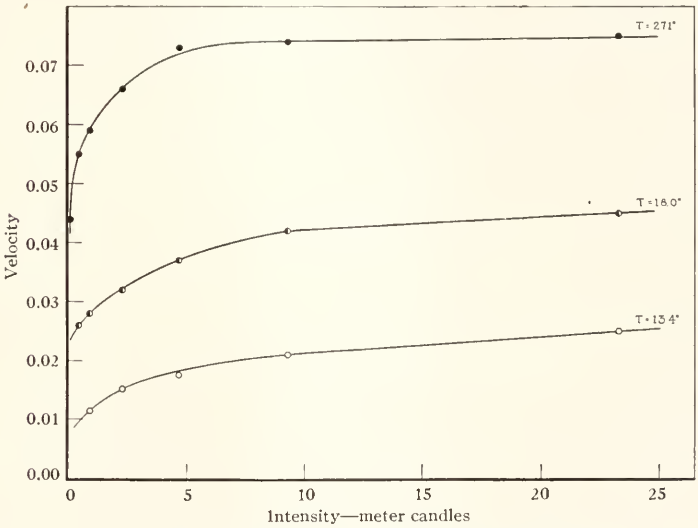

FIG. 5. Plot of data of Table IV as velocity against intensity. Open circles are Series 8.21 *A*; half-closed circles, Series 8.21 *B*; closed circles, Series 8.21 *C*. Temperatures as indicated.

of 100 at 0° C., sea water of 30 per cent salinity has a viscosity of 102, and this is reduced to 52 at 25° C. Thus sea water at 25° C. is approximately half as viscous as that at 0° C., and the same body would sink twice as fast at 25° C. as at 0° C. Viscosity changes with changes in temperature would therefore account for an appreciable part of the change in speed of swimming.

If we plot the data of Table IV as the logarithm of the velocity against the logarithm of the light intensity, we obtain as before essentially rectilinear graphs (Fig. 6), which vary in slope and in position relative to the abscissa. As in Series 8.6 (Fig. 4), the points representing velocities at or near the maximum do not fall on the lines, but over a definite range of intensities the straight line fit is good. The relative displacement of the graphs, and therefore the value of C in the expression $\log V = k \log I - C$, is seen to change considerably with a change in temperature. In addition, the slope of the lines is also seen to change. At 13.4°, k has a relative value of 0.225; at 18.0° $k = 0.175$; at 27.1° $k = 0.125$. At a given temperature the effect of increasing the intensity of illumination, within certain limits, is to increase the velocity. The changes in slope of the lines in Fig. 6 indicate the effect of temperature on the relation between velocity and intensity. At 27.1° the slope is less than at the lower temperatures, and the maximum rate of swimming is attained at a lower light intensity than at 18.0°. The expression

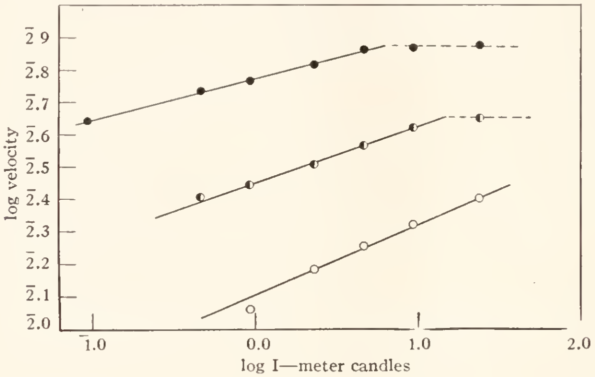

Fig. 6. Data of Table IV plotted as logarithm of velocity against logarithm of intensity. Symbols representing Series as in Fig. 5. See text.

$$\frac{\Delta V}{\Delta I} \propto \frac{V}{I}$$

implies that if V be increased by operation of a variable independent of

I, the effect of increasing I must be correspondingly less—which is the fact. We have seen that temperature and light play an important part in determining the rate of locomotion of a crustacean larva. Other factors such as age and changes in viscosity of the surrounding medium must also be taken into consideration.

SUMMARY

1. Larvæ of *Pinnotheres maculatus* Say are shown to be satisfactory animals for the study of photokinesis. The velocity of swimming is found to be greatly influenced by temperature and light intensity. Age, although not carefully investigated at present, is also an important contributing factor in determining the rate of locomotion.

2. A series of measurements of the effect of temperature on the velocity of swimming, at a constant light intensity, showed the applicability of the Arrhenius equation, and yielded values of μ of 12,800 above 18.5° and 16,900 below 18.5° C.

3. The larvæ are found to be sensitive to only a small range of light intensities. At temperatures between 20–25° C., the maximum possible velocity of swimming is attained at intensities between 10 to 25 meter candles. When velocity is plotted against light intensity a smooth curve is obtained. The same data when treated empirically according to the equation $\log V = k \log I - C$, yields essentially rectilinear graphs which are more satisfactory for a comparison of such data.

4. When series of measurements are made to determine the effect of light at different constant temperatures it is found that, besides a marked effect on general activity, there is a change in the relationship of velocity to intensity; the slopes of the curves change, and the maximum possible velocity of swimming for each temperature is reached earlier at the higher temperatures.

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BRANCHIAL RESPONSES TO ADRENALINE AND TO PITRESSIN IN THE EEL

ANCEL KEYS[1] AND J. B. BATEMAN[2]

(From the Physiological Laboratory, Cambridge, England)

INTRODUCTION

The gill perfusion method (Keys, 1931*a*) affords a convenient means of investigating the physiology of the branchial blood vessels in the fishes. In particular, it would seem to be desirable to study the effects of various hormones on the effective calibre of the branchial vessels and on the performance of the branchial chloride-secreting mechanism (Keys, 1931*b*).

We have investigated the effects of adrenaline and of pitressin on the gills of the eel, *Anguilla vulgaris,* by means of the ventral aorta-gill preparation as described in a recent paper (Bateman and Keys, 1932). Throughout each experiment the perfusion pressure (mean of systole and diastole of the pump) was maintained at a constant level by adjustment of the pump stroke and the reservoir level.

Rates of perfusion were measured from the rates of inflow in some of the experiments and from the rates of outflow from the dorsal aorta in the other experiments. In the latter cases the dorsal aorta was cannulated at the level of the anterior portion of the liver. Practically identical results were obtained from these two methods. Venous escape from the cardinal and coronary systems was prevented in all cases except where specifically mentioned.

The perfusion fluid used was the same as that given by Keys (1931*a*, p. 359), the concentrations being $\Delta =$ about 0.72° for eels from sea water and $\Delta =$ about 0.60° for eels from fresh water. The external medium was either Plymouth sea water or Cambridge tap water, depending upon the medium in which the eels had been kept prior to the experiment.

The net chloride exchange between internal and external media in the gills was determined by analyses of ingoing and outgoing fluids, using Keys' (1931*c*) method. Analyses were done in duplicate or triplicate.

The effects of adrenaline were determined by the addition of adrenaline chloride (Parke Davis) to the perfusion fluid immediately before use so as to give adrenaline concentrations between 1/300,000 and 1/1,000,000. Pitressin (Parke Davis), the pressor principle from the posterior lobe of the pituitary gland, was used in concentrations ranging from 25 to 50 international pressor units per liter by addition to the perfusion fluid just prior to use. The normal perfusion fluid was used as control in all cases.

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[1] Fellow of the National Research Council.

[2] Working on behalf of the Medical Research Council.

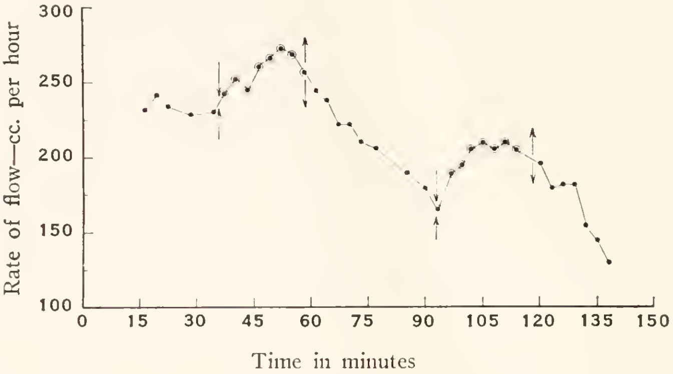

Fig. 1. The influence of adrenaline (1/1,000,000) on the rate of flow through the gills under constant pressure. The encircled points are from measurements during adrenaline perfusion, and the arrows indicate the introduction and removal of adrenaline.

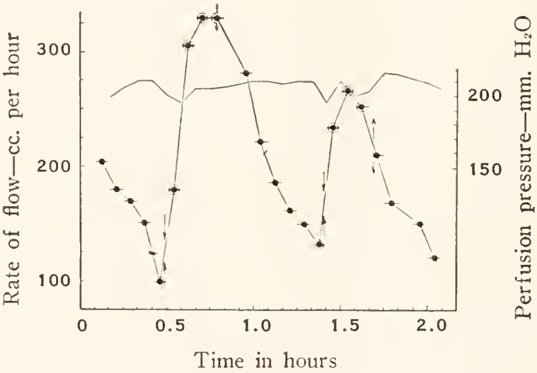

Fig. 2. The influence of adrenaline (1/500,000) on the rate of flow through the gills under constant pressure. The line drawn through the points shows the rate of flow. The encircled points are from measurements during adrenaline perfusion, and the arrows indicate the introduction and removal of adrenaline The continuous line shows perfusion pressure.

The Effect of Adrenaline on the Calibre of the Branchial Vessels

Adrenaline, in the concentrations used here, provoked unmistakable dilatation of the branchial vessels. Figures 1 and 2 show the pronounced increase in rate of flow through the vessels resulting from the addition of adrenaline to the perfusion fluid.

The experimental results shown in Fig. 1 and Fig. 2 are typical. The results of additional experiments are shown in Table I in which mean relative values for rates of flow with and without adrenaline are given. In each case the initial rate of flow without adrenaline is taken as 100 and all subsequent rates of flow expressed as percentage of the initial value.

TABLE I

Effect of adrenaline on the mean rate of flow through the perfused branchial vessels. All rates of flow expressed as percentage of the initial value.

Adrenaline concentration	Rate flow without	No. determinations	Rate flow with	No. determinations	Rate flow without	No. determinations	Rate flow with	No. determinations	Rate flow without	No. determinations	Rate flow with	No. determinations
1/300,000......	100	3	143	10	128	11						
1/500,000......	100	9	172	4	110	5	160	3	96	4		
1/500,000......	100	6	116	10	91	9	96	6	75	7		
1/700,000......	100	3	123	4	110	4	133	5	111	5	131	4
1/1,000,000....	100	5	112	7	103	4						

Adrenaline in these concentrations produces vasoconstriction in the vessels (other than the coronaries) of mammals. However, adrenaline in some concentrations (about 1/20,000,000) is known to have a small true vasodilator action (Dale and Richards, 1927) and it was thought necessary to test the effect on systemic vessels of the eel of the adrenaline concentrations used in the gill perfusions.

A preparation was made with the posterior part of the eel perfused from the dorsal aorta. The adrenaline effect was entirely similar to that obtained with the systemic vessels of the mammals, very pronounced vasoconstriction being obtained with adrenaline concentrations of 1/500,000 and 1/1,000,000. Figure 3 gives the results of an experiment of this type.

The final proof of the dissimilarity of the action of adrenaline on the branchial vessels and on those of the systematic circulation is provided by the following experiment. An eel is pithed and secured to the operating board. A mid-line incision is made exposing the heart and the ventral aorta. The sinus venosus is opened and the ventral aorta cannulated so that the course of the perfusion is through the gills to the systemic circulation and finally out through the opening in the sinus venosus. If, now, adrenaline is added to the perfusion fluid and the perfusion pressure is kept constant there is an almost immediate great reduction in the rate of flow, and removal of the adrenaline from the perfusion fluid restores the perfusion rate as soon as the adrenaline has been washed out of the system.

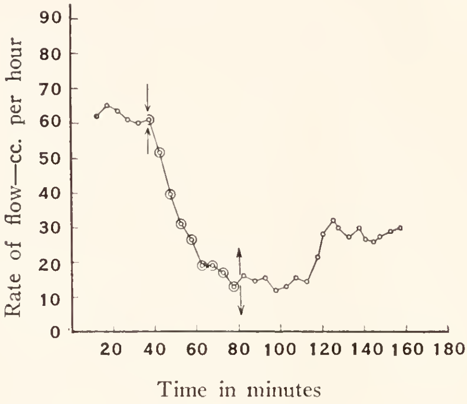

FIG. 3. The influence of adrenaline (1/500,000) on the rate of flow through the systemic vessels of the posterior part of the eel under a constant perfusion pressure of 35 cm. H_2O. The encircled points are from measurements during adrenaline perfusion and the arrows indicate the introduction and removal of adrenaline.

The effect of the adrenaline on the gills alone is demonstrated on the same preparation simply by cutting through the dorsal aorta at the level of the sinus venosus so that the perfusion is short-circuited past the systematic circulation. The results of this experiment are shown in Fig. 4.

THE EFFECT OF ADRENALINE ON THE CHLORIDE SECRETION

The apparent chloride secretion was always depressed by adrenaline in the experiments in which chloride concentrations were measured. The results of the two most complete experiments of this type are shown in Fig. 5 and Fig. 6.

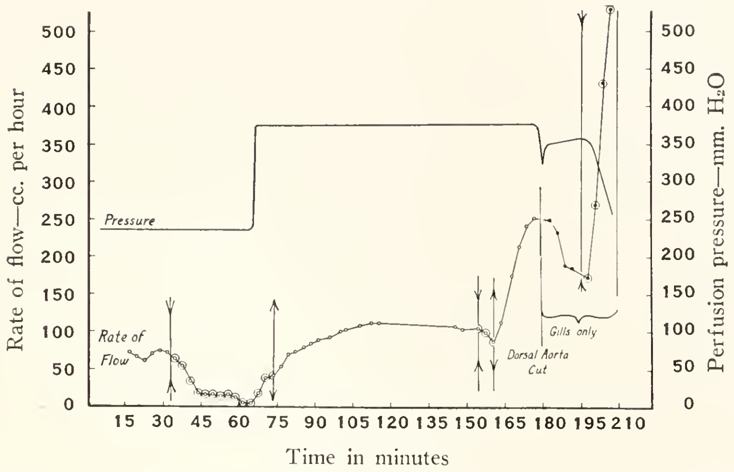

FIG. 4. A comparison of the adrenaline effect on the systemic and branchial vessels of the eel. The encircled points are from measurements of rates of flow during adrenaline perfusion and the arrows indicate the introduction and removal of adrenaline. See text for details of the experiment.

It will be noted that the adrenaline vasodilatation is very evident and closely parallels the very pronounced diminution in the chloride secretion. Figure 6 is particularly instructive; under the influence of adrenaline the secretion not only disappeared but the internal fluid became more concentrated during its passage through the gills. Table II, containing the condensed protocol of this experiment, will make clear the details of the method of calculation employed.

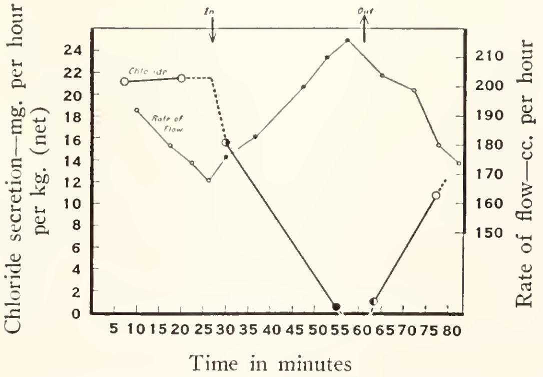

FIG. 5. The effect of adrenaline on chloride secretion in the gills bathed externally with sea water. Concentration of adrenaline, 1 in 1,000,000.

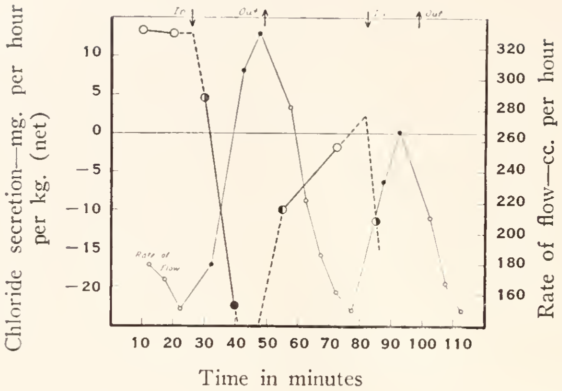

FIG. 6. The effect of adrenaline on chloride exchange in gills bathed externally with sea water. Concentration of adrenaline, 1 in 500,000.

The mean chloride change for all the experiments was 14.5 mg. in the periods with normal Ringer's solution and 1.9 mg. in the adrenaline

TABLE II

Protocol of Experiment 2A on the effect of adrenaline on the secretion of chloride by the perfused branchial vessels.

Eel from sea water, weight 370 grams. External medium, sea water; Δ = 2.0°. Internal medium (1) normal; eel Ringer's solution; Δ = about 0.72°. (2) adrenaline; same as (1) but plus adrenaline chloride to give final adrenaline concentration of 1/500,000.

Internal medium	Time of collection of perfusate and control		Mean perfusion pressure	Mean rate flow	Chloride concentrations		Difference chloride	Net chloride change
	beginning	end			control	expt.		
			mm. Hg	*cc./hr.*	*mg./100 cc.*	*mg./100 cc.*	*mg./100 cc.*	*mg./hr./kg. eel*
Normal	1:05	1:15	20	202	693.0	691.4	−2.6	−13.4
Normal	1:15	1:25	21	160	693.0	690.0	−3.0	−13.0
Adren. (in gills about 1:31)	1:25	1:35	19	189	692.6	691.7	−0.9	− 4.6
Adrenaline	1:35	1:45	20	318	692.4	696.0	+2.6	+22.3
Normal (adr. out about 1:50)	1:45	2:05	20	278	693.8	695.1	+1.3	+ 9.8
Normal	2:05	2:20	20	166	693.5	693.7	+0.2	+ 0.9
Adrenaline (in gills about 2:25)	2:20	2:30	19	183	693.1	695.4	+2.3	+11.4
Adrenaline	2:30	2:40	19	259				
Normal (adr. out about 2:45)	2:40	2:50	21	189				
Normal	2:50	3:05	20	135				

periods, these figures representing the net loss of chloride from the internal medium per hour and per kilogram of eel. These values give really a minimum estimate of the difference between the apparent secretion in the two conditions owing to the fact that there was a considerable amount of overlap in many of the collections. In almost all cases the first period of collection of perfusate following a change in the perfusion medium represented, in reality, a mixture of perfusate from the adrenaline and the normal perfusion fluids.

The Effects of Pitressin on the Gills

The experiments with the pressor principle ("pitressin," Parke Davis) of the pituitary gland demonstrated only that this hormone, in the concentrations we used, has little effect either on the mean calibre of the branchial vessels or on the branchial chloride secretion.

In four experiments involving eight periods of normal perfusion fluid and six periods of pitressin (25 to 50 units per liter), the effect of the pitressin on the rate of flow was slight and variable. The maximum effect observed was a mean *decrease* of 17 per cent; the next greatest effect was an *increase* of 10 per cent. The average of all the measurements of rate of flow was very nearly the same with the normal perfusion fluid and with the pitressin. Taking the average of the non-pitressin periods as 100, the average for the pitressin periods was 97.

The chloride secretion by the gills appeared to be almost unaffected by pitressin and such effects as were observed were variable from one experiment to the next. In two experiments there was an increase in the chloride secretion during pitressin but in two other experiments the effect was in the opposite direction. In all cases the effect was small. The maximum change in the net chloride secretion during pitressin was an increase of 5.3 mg. Cl per hour and per kilogram of eel; the next greatest effect observed was a decrease of 4 milligrams.

Discussion

Wyman and Lutz (1932) have studied the effects of adrenaline on the blood pressure of the elasmobranch, *Squalus acanthias*. They recorded dorsal and ventral aorta blood pressures and found sustained pressor effects even with very small doses of adrenaline, which they interpreted ". . . as being due to extra-cardiac factors, peripheral to the gill capillaries, but the region of action of the adrenaline was not located."[3]

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[3] Strictly, there are no true capillaries in the gills; see Keys and Willmer (1932) for the structure of the branchial vessels.

These results are obviously in complete agreement with our findings and we feel safe in predicting that suitable experimental technique would reveal adrenaline vasodilatation in the gills of the elasmobranch.

Since the completion of the present experiments we have discovered an interesting paper by Krawkow (1913) which apparently has escaped notice by other workers. Krawkow removed the gills of the pike and cannulated the stump of the ventral aorta. He estimated vasodilatation and constriction by perfusing and counting the number of drops from the cut ends of the gill bars, and he studied the effects of various substances with this technique. His results may be summarized in his own words (p. 603): "Von den untersuchten Substanzen bewirken Imidazolyläthylamin, Nikotin und Chlorbaryum Verengerung der Kiemengefässe. Coffein bewirkt nach kurzdauernder geringer Verengerung bedeutende Erweiterung der Gefässe. Chloroform erweitert die Kiemengefässe. Adrenalin, sogar in starken Verdünnungen, bewirkt sehr bedeutende Erweiterung die Kiemengefässe."

It would seem that the vasodilator effect of adrenaline in the gills of fishes is general. The similarity of this response to the adrenaline vasodilatation of the coronary vessels will have been noted. There is no longer any question as to the adrenaline vasodilatation of the coronary vessels (see Anrep, 1926, for literature), and Rössler and Pascual (1932) have shown that this is a direct response and is not due to an accumulation of metabolites. The latter possibility, of course, is eliminated in perfusion experiments like the present.

It is well known that the coronary arteries of the fish heart arise chiefly from the ventral ends of the posterior branchial vessels (Cuvier, 1805, and many subsequent workers,—see Grant and Regnier, 1926, p. 293) and, in fact, may be considered to be outgrowths of the branchial vascularization. Grant and Regnier (*op. cit.*) have assembled evidence to show that the coronaries in all probability are homologous throughout the vertebrates, ". . . the only important point of difference being the remote branchial origin in the lower vertebrates," (p. 294).

It does not seem to be unwarranted to suppose that the original specialization of vessels which respond to adrenaline by dilatation rather than constriction occurred in the gills of the early fishes. The gill most certainly began to evolve before the coronaries and it is difficult to see how an animal could survive if the vessels of its gills were subject to constriction at those critical moments of danger when adrenaline is liberated into the blood stream. If this view is correct, then the vasodilatory response to adrenaline characteristic of the coronary arteries of the higher vertebrates represents a useful heritage of the primitive condition in the gills.

There are two possible explanations of the effect of adrenaline on the chloride secretion. It may be, of course, that adrenaline simply paralyzes the secretory mechanism; the increase in the concentration of the internal medium during its passage through the gills which is observed in some cases following adrenaline (see Fig. 5) would then result from the normal permeability (Sumner, 1906; Smith, 1930; Keys, 1931*b*) of the gill membranes. This explanation is open to the objection that the indicated permeability may far surpass other estimates of the normal gill permeability (Keys, 1931*b*, p. 378 et seq.).

The alternative explanation for the effect of adrenaline on the chloride secretion is that the vasodilatation greatly increases the permeability so that the passive exchange may exceed the effect of the secretion. Such a relation between dilatation of minute vessels and permeability is apparent from the work of many investigators (see Krogh, 1929, p. 332) and would seem to be quite general. Krogh (*op. cit.,* p. 335) states: " I find no case in which a considerable dilatation has taken place without being accompanied by an increase in permeability."

We have compared the adrenaline response of the gill vessels to that of the coronary arteries; it may be instructive to make a similar comparison with regard to the effect of pitressin. Various workers have studied the effect of pituitary extracts on the calibre of the coronary vessels, but the results have not been consistent. Pal (1909), De Bonis and Suzanna (1909), and Dale (1909) reported vasoconstriction in mammalian coronaries, but Cow (1911) and Rabe (1912) could not confirm these results. Sumbal (1924) reported vasodilatation in the coronaries of the tortoise following administration of " infundin."

It seems that both the coronary vessels and the gill vessels may differ from the systemic vessels in not conforming to the general rule of vasoconstriction with pituitary extracts. It may be mentioned, however, that Drinker (1927) perfused the web of the frog with pituitrin (which is now known to be a mixture of the pressor and oxytocic principles of the pituitary gland) and found little effect on the diameter of the capillaries and no effect on the permeability.

In view of the pronounced effect of pitressin on the secretion of the kidney (Bugbee and Simond, 1928), it is of interest to note that no such effect on the branchial secretion was found in spite of certain other resemblances (Keys, 1931*b*, p. 382 et seq.) between the performance of the two organs.

SUMMARY

Perfusion experiments are described in which the effects of adrenaline and of pitressin on the gills of eels were studied.

It is shown that adrenaline has a marked vasodilator effect on the gills whereas the same concentration of adrenaline has a powerful constrictor effect on the systemic vessels.

Adrenaline was found to decrease or abolish completely the branchial secretion of chloride; this effect is interpreted as being due to increased permeability of the gills associated with vasodilatation.

Pitressin did not appear to have any large or consistent effect either on chloride secretion or on the effective calibre of the branchial vessels.

. The evolutionary and physiological relations between the gills and the coronary vessels are discussed. It is suggested that the adrenaline vasodilatation of the coronary arteries may represent a phylogenetic inheritance of a specialized vascular response originally developed in the gills.

Our best thanks are due to Professor Joseph Barcroft for his interest in these studies.

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THE

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THE FOURTH REYNOLD A. SPAETH MEMORIAL LECTURE[1]

Genetics and Development

RICHARD GOLDSCHMIDT

Kaiser-Wilhelm-Institut für Biologie, Berlin-Dahlem, Germany

It is one of the sad privileges of men advancing in age to be eligible to the honorable task of delivering lectures dedicated to the memory of a much younger friend whom fate has not permitted to fulfill the great expectations held for him by those who knew him best. In accepting the honor to deliver this memorial lecture, I quite naturally recall the Woods Hole days sixteen years ago, when Reynold A. Spaeth was one of the few with whom I used to discuss certain questions which then were uppermost in my mind. The selection of this evening's topic is therefore influenced by these recollections.

During the summer of 1916 I had the honor to deliver right here in the old lecture hall an evening lecture, in which I tried to explain the experimental results of my work on intersexuality, a term which I had introduced only a year before. I proceeded then to derive from the facts a general theory of sex-determination, which I had developed since 1911, but which had not yet come to be known in this country; a theory which nowadays is called the theory of the genic balance of the sex genes. At the end of this lecture I hinted with a few words at further consequences of the analysis of intersexuality. According to the printed report in the American Naturalist of 1916,[2] I said: "Very important new facts will be published later which will probably enable us to replace the symbolistic Mendelian language, used here, by more definite physico-chemical conceptions." And further: "I am rather optimistic in regard to the general conclusions which might be drawn from these facts, as well as regards the sex-problem as on some fundamental questions of heredity. Combining these facts with the work on hormone action as related to sex, we can, I think, form a pretty clear idea about sex differentiation and determination. If we put them in line with the facts of experimental embryology concerning the determination problem we see the outlines of a promising theory of heredity."

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[1] Delivered at the Marine Biological Laboratory, Woods Hole, on August 19, 1932.

[2] Experimental Intersexuality and the Sex-problem. *Am. Nat.*, **50** (1916).

During the many years which have since passed, I have tried to formulate the conclusions at which I hinted then and to find new experimental evidence on which to base them. And still, after much thinking on the subject, I stand by the words quoted from 1916, namely: "I am rather optimistic in regard to the general conclusions, etc." The more facts are being accumulated and the more I try to coördinate them and to see a simple guiding idea behind their diversity, the more I am convinced that my method of general approach, which has been highly praised by some and severely criticized by others, is the only one which leads to a deeper insight into the process of heredity. This then is the reason why I have not chosen to present here today some of my recent experimental work, but rather to continue some of the general reasoning from the point where I had left it in my lecture of sixteen years ago.

The decisive step in the analysis of intersexuality, which geneticists often found difficult to understand though physiologists were usually willing to accept it, was that step which led from the static Mendelian analysis of the problem to the dynamic viewpoint of the physiology of development. Here then is found the natural point of departure for our discussion. The limits of ordinary Mendelian analysis, as known at that time, were first reached when it was shown that the experimental facts regarding intersexuality could be expressed not by a simple Mendelian formula, but only by assuming that two genes or completely linked sets of genes,[3]—those for femaleness and maleness,—controlled the result according to their quantitative relation or balance. The simple Mendelian formulation was thus enlarged by a new conception, namely that of a quantitative relation or balance of genes working together towards the production of a phenotype, the character of which was in some way proportional to that quantitative relation of the genes in question—or in other words, their amount of balance or unbalance. This new conception, which had to be added to the general Mendelian formulation and which had given me the clue to the whole analysis already at the beginning of the work between 1911 and 1914,[4] could still be expressed in the old Mendelian language, if the gene in favor of which the balance acted was called epistatic to the other and if the different degrees of this balance, to which corresponded the sexes and the different types of intersexes, were expressed in terms of degrees of epistasis, which might be measured by some unit. Thus the formulae with numerical values of the genes, symbolizing the grades of their effect, had to be introduced. It took many geneticists a long time to understand this.

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[3] The problem whether only individual sex-determiners or a completely linked group of such are involved in our case, has been repeatedly discussed, *e.g.*, Untersuchungen über Intersexualität V. *Zeitschr. f. indukt. Abst. u. Verer.*, **56** (1930); Analysis of Intersexuality in the Gipsy Moth. *Quart. Rev. Biol.*, 1931.

[4] The whole literature on the subject is found in the author's book: "Die sexuellen Zwischenstufen," J. Springer, Berlin, 1931; further in the paper quoted in footnote 3.

But still another extension of Mendelian language was necessary to cover the facts. If the different amounts of the unbalance of male and female genes were to stand for the normal sexes as well as for the different degrees of intersexuality, it followed necessarily that a certain minimum value of this balance had to exist below which one of the pure sexes was determined, and another maximum value, above which the other sex was determined, the intersexual stages lying between. These limiting values for the balance of female and male genes were accordingly called the epistatic minimum, a term which again meant a necessary extension of ordinary Mendelian conceptions, in order to describe the experimental facts still in the language of static Mendelism. This was the point reached in 1912, a point which was situated at the utmost limits of purely Mendelian conceptions. This became clear when the fact was considered that there existed two completely different types of intersexes, namely male and female intersexes, which replaced in the respective experiments the gametic males or females. Now the Mendelian formulation which had covered the case thus far by the introduction of the principle of genic balance and of the epistatic minimum could describe adequately the production of a series of intersexes between the two normal sexes, that is the two limiting minima, but it could not explain why the same ratio between male and female determiners, say the one midway between the ratios for the normal sexes, determined in one case a medium grade female intersex and in another case the completely different medium grade male intersex. Here then was the point at which the power of static Mendelism ended and further progress was only possible by the transition to a dynamic point of view; in other words, the genetic explanation was to be followed by one based upon the physiology of development.

This step, at which I had hinted in the previously mentioned evening lecture given here at Woods Hole, could be taken when it was found what these intersexes really were. It became apparent first in 1916 (and as a matter of fact I do not understand now why I had missed this point in the preceding years) that in a series of intersexes connecting the two pure sexes step by step, such organs which are the last to differentiate in development are the first to assume the character of the opposite sex in the case of low grade intersexuality, and that, vice versa, the organs which are the first to differentiate in development are the very last to change towards the other sex in high grade intersexuality. From this rule it followed that intersexes are to be considered as individuals which have begun their development as of one sex up to a certain turning-point and have finished it as of the opposite sex after the turning-point;[5] further, that male intersexes begin as males and end as females and that female intersexes begin as females and end as males; and further, that the different grades of intersexuality are a function of the position in time of the turning-point; earlier turning-point—higher grade of intersexuality. This solution which I also had the pleasure to announce first in this country, namely at the 1916 meeting of the American Association, has meanwhile been tested by extensive embryological study and found to be an actual fact. It opened now the way to the solution of the whole problem by connecting a definite embryological process with a definite genetic condition.

The situation was this: On the genetic side we had first a gene or genes for maleness, second a gene or genes for femaleness and both in a series of different conditions, found in different races; further we knew that the phenotypic effect of these genes, namely maleness, femaleness, and all degrees of intersexuality, was proportional to the amount of balance or unbalance of these genes. On the embryological side, we had the occurrence of the turning-point for sexual differentiation at a definite time, and combining now the genetic side with the embryological side, we were facing the fact that a series of increasing values for the unbalance or abnormal ratio of the sex genes has its effect in a series of corresponding changes in the time of incidence of the turning-point, which occurs earlier and earlier. Here then was an opportunity to connect the action of definite genes, present in different ratios, with an embryological event, occurring at definite and proportionally different times. Whereas we have genes for both sexual differentiations simultaneously present, and whereas the control of actual sexual differentiation belonged first to one and later to the other gene or set of genes and whereas this control changes at a definite time, which is conditioned by and proportional to the unbalance or ratio of these genes, there is left only one way of linking these facts together: namely, by concluding first, that the genes in question are producing each independent chains of reaction which lead at a certain threshold to the production of the something which controls sexual differentiation; second, that the amount of unbalance of the two genes or their ratio results in corresponding different velocities of the two chains of reaction; third, that the reaction of higher velocity controls the sexual differentiation; and fourth, that the turning-points therefore must be the points at which this control changes, which means graphically points of intersection of the two respective curves of reaction. Standing in this place here I cannot help recalling Jacques Loeb's excitement when I told him this story and some of the consequences regarding a general theory of heredity. I have since discussed this point with other great physiologists who agree with me that there is no other way of representing the actual facts from a dynamic point of view. Thus I concluded that here a case was found in which the action of definite genes could and had to be interpreted in terms of speed of reactions and that it might be possible to base a theory of genic action upon this interpretation. Also this conclusion I had the pleasure to announce in this country at the said 1916 meeting of the American Association.

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[5] As a matter of fact, Baltzer had found already two years before the same for the intersexes of *Bonellia*, a fact which had escaped me for a long time.

There was also another conclusion which had to be drawn from the same facts, and with this we are getting into deep waters. The phenotypic result (male, female, male intersex, female intersex of any grade) was found to be dependent upon a quantitative relation, balance or ratio of male and female sex genes, and the genetic results showed and have ever since shown that only one female and one male gene are involved. But of each of these sex-genes a considerable number of conditions, in genetic language of multiple allelomorphs, were found which gave typical but different effects. These conditions, which proved to be absolutely constant in all experiments involving the same genes, might be termed the strength of action, or the potency or the valency of these genes, and thus the phenotypic result in regard to sex was dependent upon the relative valencies of the female and male determiners present at fertilization. Then it turned out that one of these determiners, namely the one for maleness, was situated within the X-chromosome; the other one, for femaleness, being outside the X-chromosome. This meant that the always constant genes for femaleness were faced either by one or by two genes for maleness. Thus on one hand, the genotypic effect was produced by the relative valencies of the two types of sex-genes; on the other hand, two of the possible phenotypes, namely the pure sexes, were dependent upon the ratio between the always constant female genes and the male genes present in one or two quantities. In these limiting cases, then, the pure sexes, the relative valencies, responsible for the phenotype, were obviously identical with relative quantities of these genes. But the normal sexes were only two points in a continuous series of sexual conditions, all dependent upon different relative valencies of these genes. The conclusion, therefore, was not only logical but also inevitable that all the other conditions for the sex-genes, their different degrees in strength or valency, were also in reality differences in their quantity. Thus the quantitative relation or balance of these genes finally was resolved into the ratios of actual quantities. And the former conclusion, which showed that the sex-genes acted through chains of reaction of different but typical velocities, could now be enlarged by the addition that the speed of these chains of reaction is proportional to the quantities of the genes in question.

It is a strange fact that this conclusion was regarded by some orthodox geneticists as a most condemnable heresy. To be sure, they could not give a different explanation of the facts and they could not contest the logic of the analysis. Therefore they simply declared it to be inadmissible—this word has actually been used—to assume that a gene may have a definite and fixed quantity as one of its properties and that the effect of a gene might be in some way proportional to its quantity. In our object, there was no possibility of demonstrating visibly such a difference in quantity, because in our case everything happened within the normal diploid number of chromosomes. But some experiments have since been performed with our material, besides the visible demonstration in triploid intersexuality, discovered by Standfuss and since analyzed in moths and in *Drosophila*. Our experiments in question demonstrate clearly the logic and the soundness of the conclusions.[6] To mention only one: Two X-chromosomes and therefore two male genes (in the case of female heterogamety) are determining the male sex. If, however, I combine female determiners coming from a strong race, that is genes of high valency, with two X-chromosomes derived from races of very low valency of the sex-genes, the resulting individual will be a female in spite of its two X-chromosomes. By appropriate crosses I might now build up individuals which contain the same strong female determiner as before, one X-chromosome with the very low male determiner as before but the other X coming from a race with a little higher grade of valency of the male determiner. The individual thus composed will be a little more male than before, and this is a high grade intersexual male, very near to complete transformation into female. Now I continue replacing the second X-chromosome by one derived from a still stronger and stronger race; correspondingly, the individual in question will be less and less intersexual, so that when a certain combination is reached it will be a normal male. Table I gives an actual experimental result. Now this experiment and its easily imaginable variations show that the action of the two male genes which are present in any case is proportional to the sum of the valencies of the two genes. As a matter of fact, we ought to be able to calculate from a series of such experiments the relative valencies of all these genes in some arbitrary units, because these experiments furnish a number of equations which may be solved. Thus we have a number of differently active genes and any two of them act together always in proportion to their sum. I can draw from this no other conclusion but that it is the quantity of the thing in question which determines its action.

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[6] Details are found in Untersuchungen über Intersexualität, I–V. *Zeitschr. f. indukt. Abst. u. Verer.*, 1920–30; see especially No. V.

I have never been able to understand why this conclusion, which safely rests on experimental facts, has been considered by some as offensive. The number, the size, and the shape of the chromosomes are constant; the size of cells is constant and often their number in a given organ; the number and size and arrangement of blastomeres are constant, the number of segments, of bristles, and I know not what else.

TABLE I

Strong F from race	Very weak M from race	Second M from race	♂	Intersexual ♂ grade						♀ (gametic ♂)
				I	II	III	IV	V	VI	
Tokyo	Hokkaido	Hokkaido								—
"	"	Berlin							—	—
"	"	Russia					—	—		
"	"	Korea			—	—	—	—		
"	"	Kumamoto	—	—	—	—				
"	"	Kyoto	—	—	—					
"	"	Tokyo	—							

Orderly development of a given organism requires a wonderful amount of quantitative constancy from the organ down to the chromosome. Why then should exactly that bit of substance which after all is responsible for all the rest be required to produce its wonderfully typical action of a unique sameness on the basis of a negligible quantity? To my mind, even apart from all the evidence produced, the first requirement for something like an understanding of the action of a thing like a gene would be its presence in typical quantity at the onset, because the mass of a reacting substance is always the first variable to be considered. If in addition, the facts reveal such a simple relation as that between the quantity of the reacting gene substance and a corresponding velocity of reaction, I am ready to consider this as a fundamental insight, upon which one ought to be able to build a theory of the genic action, a theory of heredity.

I have tried now to show how step by step the results of my experiments forced me first to stress the purely Mendelian conceptions by introducing the idea of genic balance and of the epistatic minimum and then to go beyond the limits of static Mendelian conceptions towards the goal of a dynamic understanding of a gene-controlled determinative process. The next step to take was naturally to try to apply the fundamental conceptions to the elaboration of a general theory of heredity, based on the principle of coördinated reaction velocities, as announced in my lecture here sixteen years ago.

It is only recently that I learned[7] that a few years before I had derived my conclusions and had embarked upon their generalization, Professor M. F. Guyer had already arrived at a similar conception which, though no experiments were available at that time, was developed by him in a very ingenious way. I am glad to make use of this occasion to pay my respects to Professor Guyer's intuition and to quote some of his sentences, namely:

"If in the comparatively simple cases of associated simultaneous reactions with which we are acquainted in non-living matter, relative velocities may so modify the results, we can readily realize of what tremendous importance regulation of this matter must become in living protoplasm where doubtless vast numbers of chemical reactions and interactions are going on at the same time. In fact, could we locate such a time-regulating factor in the germ-cell it would seem that we had accomplished a long stride toward an understanding of the controlling and coördinating mechanism which insures the appearance of just the proper substance at the right time in morphogenesis. It would constitute a qualitative as well as a quantitative regulator, for by determining quantity at any given time it determines what the next chemical reaction will be, and hence in the very doing of this, it necessarily conditions the chemical outcome of that reaction."

There can be no doubt that these sentences contain already the essence of the theory of the orderly arranged, interwoven, and balanced velocities of reaction. Returning now to the further development of my own work, I obviously continued arguing the following way: Determinative processes in regard to sex have to do with almost any type of morphological and physiological differentiation occurring in development. If, for example, we turn our attention to a single organ like the genital armature in insects, which exhibits differences in the two sexes, of a degree, which might be compared to the differences in structure of two far distant organisms, we realise the amount and diversity of specific differentiation which may be brought about by such a simple system of coördinated reaction velocities as that which had been actually demonstrated. And if we include in this deliberation all the complex forms of one and the same organ which are obtained in a thoroughly orderly fashion in case of intersexuality, which means in consequence of a change in the coördination of the system of reaction velocities, we come to the conclusion that a similar conception ought to be applied to all types of morphogenetic processes, that is, to development in general. Development ought to be disentangled into a series of coördinated reactions of definite velocities, producing at a certain threshold a certain event, say the appearance of embryonic hormones or of determining stuffs, thus securing the order and seriation of developmental processes. And just as in the intersexuality experiments the genes in question controlled the respective speeds of reaction, so in normal development would the genes also control the speed of reactions with which they are concerned. Expressed more specifically, the genes must be things which produce their typical effects by catalyzing chains of reaction, the speed of which, ceteris paribus, and given the specific substance of each gene and the plasmatic substratum, is proportional to the quantity of the gene and therefore fixed within the entire system of simultaneous coördinated reactions of different speed.[8]

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[7] Guyer, M. F. The Germinal Background of Somatic Modifications. *Science*, 71 (1930).

We have tried since to demonstrate in detail how such a system accounts not only for numerous genetic facts, but also for facts of experimental embryology; and indeed even sheds light on evolutionary questions. I shall not try now to develop these conclusions, as it is my intention this evening to discuss in the first line the experimental and logical basis of the whole argument. The principle will, moreover, be visible incidentally if I continue relating the actual sequence of findings which helped to shape these ideas. The different sex-genes of typical valency or quantity behaved in the experiments as a series of multiple allelomorphs, of which 8–10 members have been isolated by now.[9] Simultaneously I was studying another series of multiple allelomorphs which permitted the analysis of the effects of the genes within this series in a dynamic way, because the effect of these genes became visible in the larvae of *Lymantria*.[10] There were found races in which young caterpillars were dark and remained so through all instars. There were others which had light markings and which remained light through all instars. And there were again others which were light in the young stages and turned dark in later instars. Between these extremes all transitions were found as the curves of pigmentation show, and each of these types is produced by a member of a series of multiple allelomorphs.

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[8] These views and their consequences have been developed in: Die quantitative Grundlage von Vererbung und Artbildung. *Roux's Vorträge u. Aufsätze Entw. mech.*, **24** (1920). A more detailed account, leaving out the evolutionary side, is found in: "Physiologische Theorie der Vererbung," Springer, Berlin, 1927.

[9] Final data in: Untersuchungen zur Genetik der geographischen Variation III. *Roux's Arch. Entw. mech.*, 1932.

[10] Short accounts of the main facts were published in: A Preliminary Report on some Experiments concerning Evolution. *Am. Nat.*, **52** (1917); and: Die quantitative Grundlage etc. (see footnote 8). A detailed report is found in: Untersuchungen zur Genetik der geographischen Variation. I. *Roux' Arch. Entw. mech.*, **101** (1924); Also, II, Ibid., **116** (1929). Consult this for photos and curves.

A closer study of the facts then reveals that each allelomorph of the series is responsible for a process of accumulation of dark pigment on the basis of light markings, a process which proceeds with a definite velocity which is typical but different for different allelomorphs of the series, as may be demonstrated in a diagrammatic curve.[10] Here then we found again a series of multiple allelomorphs connected with a series of reactions of different velocities, and we concluded that also this series, and, perhaps, most similar series, must consist in one and the same gene in different quantities. In this case, of course, the conclusion rests on analogy, and no way to prove it is apparent. This case, however, furnished another fact which pointed in the direction of the general theory. If we cross the always light race with the always dark race, the young F_1 caterpillars are first light, but later they become dark. In Mendelian language, light was first dominant and later dark. If we remember the last curves, it is clear that the curve which is midway between the ones of the light and dark races has exactly this type, first light, later dark; and as a matter of fact the intermediate allelomorphs of the series also produce the same effect as observed here in the hybrid. This then shows clearly that dominance, recessivity, and change of dominance are here the phenotypic effects of the type of reaction curve within the whole system. From this fact then may be derived a few theoretical cases which simultaneously are apt to serve as a model for the whole generalization.

Let us consider what dominance might mean within a system of genes which are responsible for reactions with velocities in proportion to the quantity of the genes. Figure 1 [11] assumes that we consider two allelomorphs, each producing a reaction of different velocity represented by straight lines. At a certain level or threshold marked by the line M, the determinative reaction takes place. Let us now assume that we are dealing with the size of an organ which is the result of a given number of successive cell-divisions. The reaction in question may stop the cell divisions and therefore the resulting size of the organ will be smaller and smaller, the more early the reaction curve reaches the level M. If the cell divisions proceed with equal time intervals and if the reaction velocity for the heterozygote is per definitionem intermediate between the two parents, the size of the organ will also be intermediate. Let us now assume that the cell divisions in question proceed first slower and then faster, as represented on the line M_1; the same system leads then to almost complete dominance of the greater size; if, however, cell divisions proceed first faster and then slower as represented on line M_2, we find almost complete dominance of the smaller size.

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[11] Taken from "Physiologische Theorie der Vererbung," 1927.

I think that this diagram which follows immediately from the preceding analysis, is rather instructive. It demonstrates a simple interpretation of dominance; furthermore, we have to assume that the three forementioned types of cell division are themselves determined directly or indirectly by the action of other genes, which in genetic language are usually called modifiers. Dominance then is the result of the interaction in time of the heterozygous main gene with a number of others, the modifiers. Those among you who are acquainted with Fisher's so-called theory of the origin of dominance will realize at once that only such a system, as presented here, will allow that dominance is changed

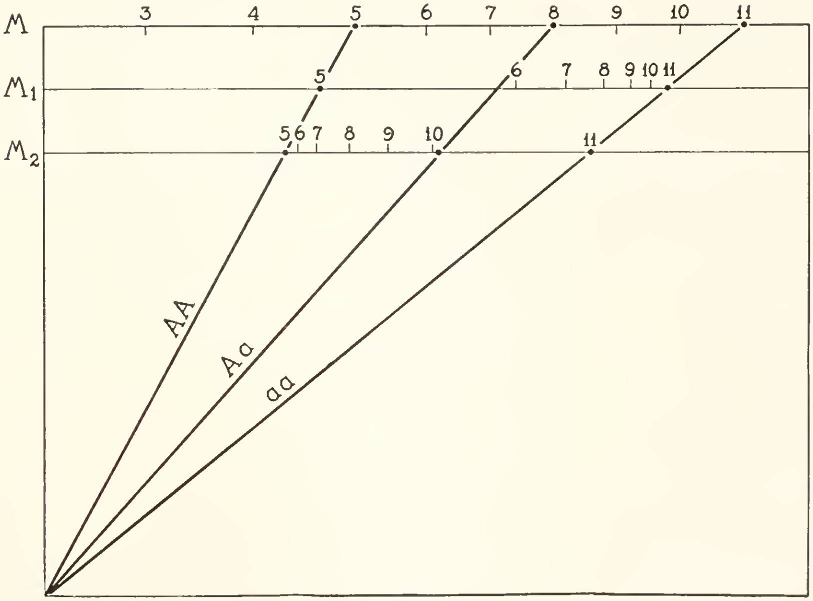

FIG. 1

by selection of modifiers. Moreover, the diagram may be used as a model for all possible determinations of developmental processes into which the embryology of an individual might be dissolved. By changing the meaning of the variables, introducing new ones, or other threshold conditions, similar models might be derived for all kinds of facts relating differentiation to genic action. Finally, the diagram may show that it is of no use to discuss the problem of the quantity of the gene without considering the corresponding reaction velocities through which alone the assumption of different but typical gene-quantities becomes important; because without this connection we have only a sterile hypothesis. Let me illustrate finally this point by an actual case. Dobzhansky[12] some time ago set out to disprove the quantitative nature of multiple allelomorphs in the following way: He argued that if we consider a series of multiple allelomorphs which produces manifold phenotypic effects in different organs, these effects must show always a parallel seriation in different combinations of these allelomorphs, if the genes in question form a quantitative series. A study of the facts did not prove this to be the case, and therefore he concluded that the allelomorphs cannot be of a simple quantitative nature. As a matter of fact the premises of this argument are already wrong, because the main point has been neglected: namely, the system of reaction velocities. This will be evident at once if we consult again a similar diagram as before (Fig. 2). We have represented three allelomorphs by their reactions of different velocities which lead to a determinative effect at a certain threshold after the times I, II, III. Let us assume again a very simple type of effect, namely the cessation of growth of an organ at the time in question. Each organ of which the size is influenced by the series of allelomorphs may, of course, have its own curve of differentiation which is determined independently of the allelomorphs in question. In order not to complicate the diagram, some of such possible curves have been drawn below. The size of the organ reached at the decisive times I, II, III is then represented by the verticals Ph I, II, III. In the first case, the organ shows a steady increase with the three allelomorphs in question; in the second organ the first two allelomorphs produce the same effect; in the third organ the effect is identical for the second and third allelomorph. The fourth case represents the growth of an organ in two dimensions represented by a length-breadth index. *L* is the curve for a constant growth in length, *W* the curve for intermittent growth in breadth, and the proportion of the two verticals at time I, II, III the respective index of the resulting phenotype. In the case which is represented, this index is first high, then low, and then again higher. This simple diagram shows then how in such a system of timed reactions a series of causes of a definite order, for example a set of different quantities of a gene, might produce effects of a very different order in different organs.

A third example of the application of the general idea might be discussed which is to be regarded as representative for a certain group of problems. The wing of butterflies and moths constitutes after a certain critical period, which is situated towards the end of the larval stage, a self-differentiating system. A nice demonstration of this I was able to

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[12] Dobzhansky, Th. The Manifold Effects of the Genes Stubble and Stubbloid in Drosophila melanogaster. *Zeitschr. f. indukt. Abst. u. Verer.,* **54** (1930). Goldschmidt, R. "Bemerkungen zur Kritik der quantitativen Natur multipler Allele." Philiptschenko Gedächtnisband, Leningrad, 1932.

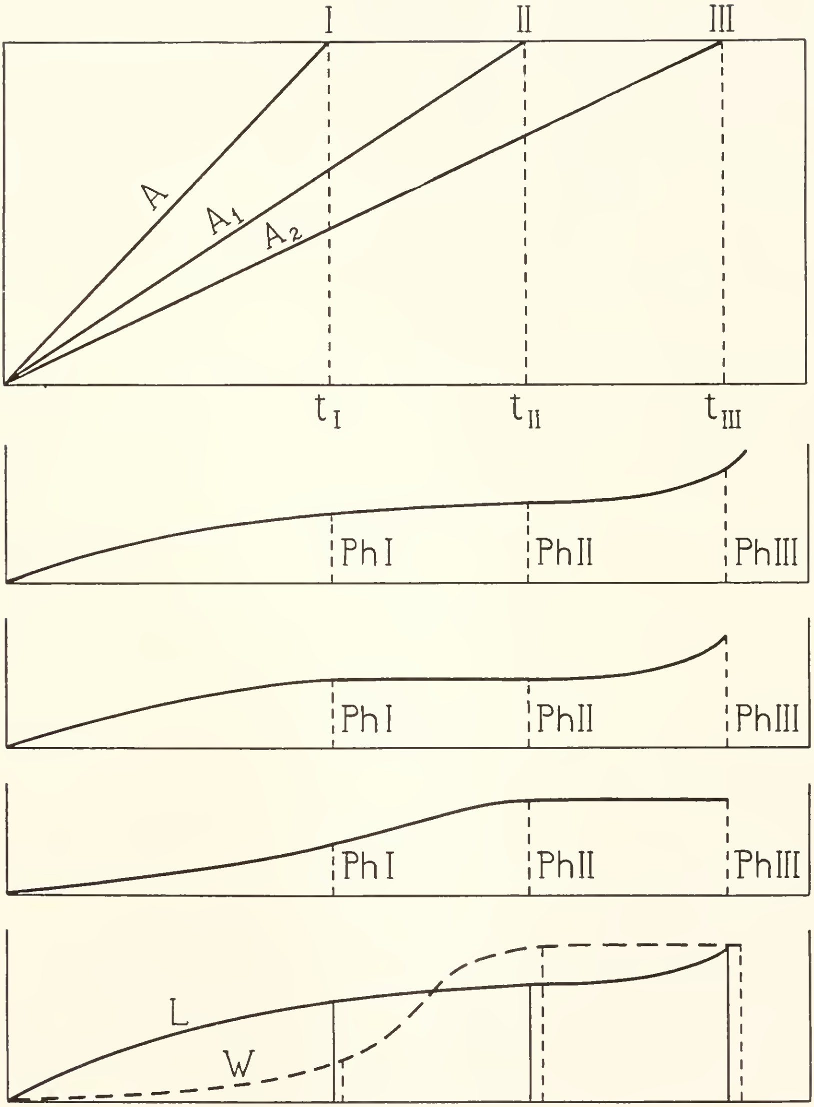
I
II
III
A
A1
A2
tI
tII
tIII
PhI
PhII
PhIII
PhI
PhII
PhIII
PhI
PhII
PhIII
L
W

Fig. 2

give many years ago[13] when I showed that it is possible to change the speed of differentiation of one wing without altering the other wing of the same individual at all, namely by blocking the blood supply to a certain extent. We may have side by side the normal wing which has almost finished its differentiation and the operated wing of the same animal which shows structure and coloration of an earlier larval period. (These experiments, by the way, have anticipated the general type of some recent experiments performed on amphibian eggs with local temperature changes.) This self-differentiating system of the wing pattern is finally determined during the critical period. At this time, when the wing is a simple epithelial sac showing no visible differentiation on its surface which would correspond to a later pattern, the future pattern is already completely laid out. How this is done we do not yet know. But two significant facts have come to light which may be regarded as the beginning of an understanding. One relates to the wing of intersexual males of the gipsy-moth. Such a wing exhibits the characteristic mosaic streaks of white female colour upon the brown male wing. If these white patches are large, it can be shown that they have also a different rate of growth from the brown areas. At the time of pupation, of course, no such structure can be seen on the epithelial wing, but in some cases the wing-mosaic may be faintly but clearly seen on the pupa-case, which has been secreted by the wing epithelium. This shows that the pattern is already present in the form of some difference in regard to the secreting activity or some other process involved in the formation of chitin by the wing-surface. A little later, however, but a long time before any pigment appears, the difference in question can be made visible, and it may be shown in what it really consists. That is, the prospective white parts of the wing are far in advance of the later dark parts in regard to the differentiation of the scales. This may be made visible by drying the wing which has been taken out of the pupa. The prospective white parts carry well-chitinized scales which remain erect when drying; the future dark parts, however, are still carrying younger soft scales which collapse in drying, so that on a wing treated in such a way the future white parts stand out in relief.[14] This then shows in one case that the primary pattern formation consists in producing areas with different speed of differentiation. These findings in the intersexual wing proved further to be in full harmony with other results in regard to the development of the wing pattern, which had been found in other objects. I could show that the normal wing pattern in many different types of butterflies and moths is laid down in the same way,[15] namely, as regions of different speed of differentiation.

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[13] Untersuchungen zur Entwicklungsphysiologie des Flügelmusters der Schmetterlinge. *Arch. Entw. mech.*, 47 (1920).

[14] For particulars and discussions see: Untersuchungen über Intersexualität. II. *Zeitschr. f. indukt. Abst. u. Verer.*, 29 (1922). Einige Materialien zur Theorie der abgestimmten Reaktionsgeschwindigkeiten. *Arch. Entw. mech.*, 98 (1923). The majority of the extensive studies of the author and his former student F. Süffert have never been published. The same principle has been always found at work.

The following slide showed a swallow-tail *Thais polyxena* with its characteristic pattern and besides a wing taken from a pupa, before any pigment becomes visible. In drying the wing, the ghost-pattern becomes visible because the future light scales remain erect, but the future pigmented scales collapse. The photograph does not allow it to be distinguished clearly that no pigment at all is involved in this picture. This is more easily visible in another picture representing an unpigmented pupa wing of a Cecropia moth. The white margin of the eye spot is easily seen as a group of erect scales, whereas those of the dark spot are collapsed.

The second important fact has recently been found by a student of Professor Kühn.[16] In the larval wings of the meal-moth at about the critical period he found zones of intense mitotic divisions, which corresponded to later elements of the wing pattern, elements which later follow the same law which we just described. Though it is not yet possible to coördinate and to understand all these facts, they might be represented in general terms at present in the following way: In the critical period which corresponds to the time of irreversible determination found in each study of developmental physiology of any organ, a pattern appears of physiologically different areas on the wing, different in regard to their growing activities and to their relative speeds of differentiation. This suggests the appearance and typical distribution of something like a growth hormone. All the rest of the differentiation of the pattern, however, is nothing but the consequence of a coördinated system of reaction velocities in regard to differentiation and also to chemism. Figure 3 may serve as a model for the whole process, which might be varied indefinitely to fit individual cases. We assume that the wing area differentiates during the critical period into three different parts, according to what we have seen before. Each of these areas, I, II, III, begins to differentiate at a different rate represented by the three curves T_1, T_2, T_3. At the level of the points T_1, T_2, T_3 the respective scales have reached the stage or threshold which permits of the deposition of pigments. We then see three independent gene-controlled chains of reaction which are supposed to result in the formation of some component, requisite for the final deposition of yellow, red, and black pigment respectively within the scales at the times Tp_1, Tp_2, Tp_3. Now at the time

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[15] Papers quoted in footnotes 13 and 14, see further: "Physiologische Theorie der Vererbung."

[16] Köhler, W. Die Entwicklung der Flügel bei der Mehlmotte Ephestia Kühniella Zeller, mit besonderer Berücksichtigung des Zeichnungsmusters. *Zeitschr. f. Morph. u. Okol. Tiere,* 24 (1932).

Tp_1 only the area *I* is ready to receive the stuff P_1, and therefore only this area will contain yellow scales; similarly for the two other areas. It is clear that this diagram, which is based on the actual facts, may be varied to fit any type of pattern, pigment etc., and that it might be as well used as a model for many processes of determination which after all are nothing but formations of patterns.

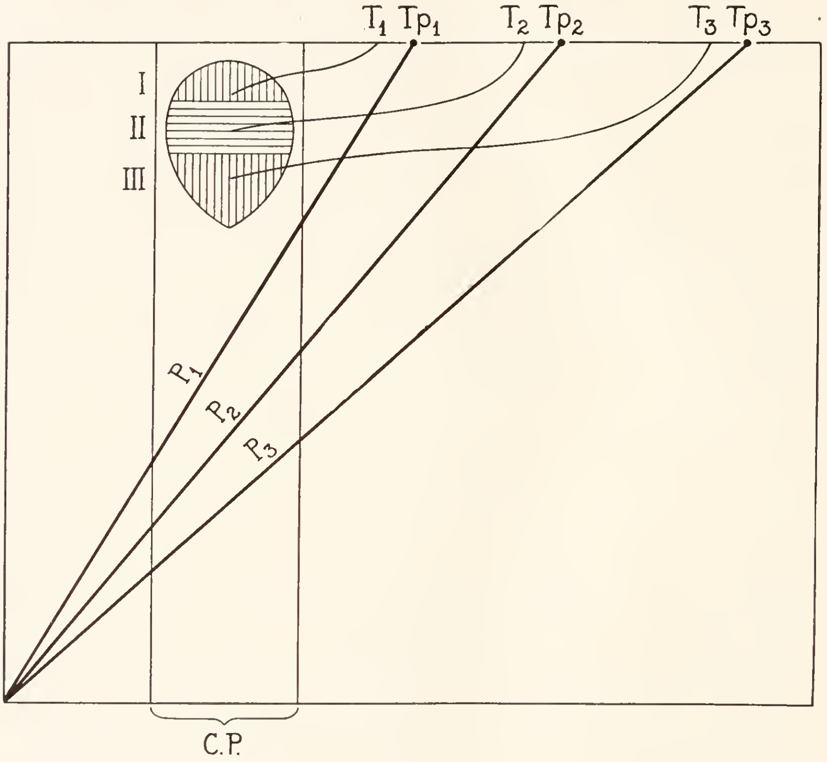

FIG. 3

Only one of the consequences may be mentioned, partly because it is connected with some of our own work, partly because it opens up vistas in another direction. The classic temperature experiments with butterflies have shown that it is possible to change the inherited wing pattern by applying extreme temperatures and other extreme conditions to the animal within the critical period, the duration of which has been exactly determined.[17] One of the well-known results of this old work, which we have repeated on a large scale, is the fact that in a number of cases it was possible to produce in the temperature experiments forms as non-heritable modifications, which are phenotypically identical with well-known geographic subspecies, a fact which plays a considerable rôle in Lamarckian discussions. A typical case is that of *Vanessa urticae* from the European continent and the subspecies *V. ichnusa* from Mediterranean islands; the phenotype of the latter is exactly reproduced in the temperature experiments with the former. Many similar cases are known. Figure 4 gives the type of explanation of such cases,[18] I repeat, the type, because no actual analysis has been made which would show which individual reactions are concerned with the special case. The diagram, therefore, does not claim to cover the actual case but to represent the type of explanation which has to be applied, all details being indefinitely variable to fit the individual case. We assume that the phenotypic differences of the two forms in question are differences in the area which one definite element of the pattern occupies. This rela-

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[17] The well-known work of Standfuss, Weismann, Fischer, Merrifield. Determination of the critical period by my former student F. Süffert: Bestimmungsfaktoren des Zeichnungsmusters beim Saison-Dimorphismus von Araschnia levana prorsa. *Biol. Centrbl.,* 44 (1924)

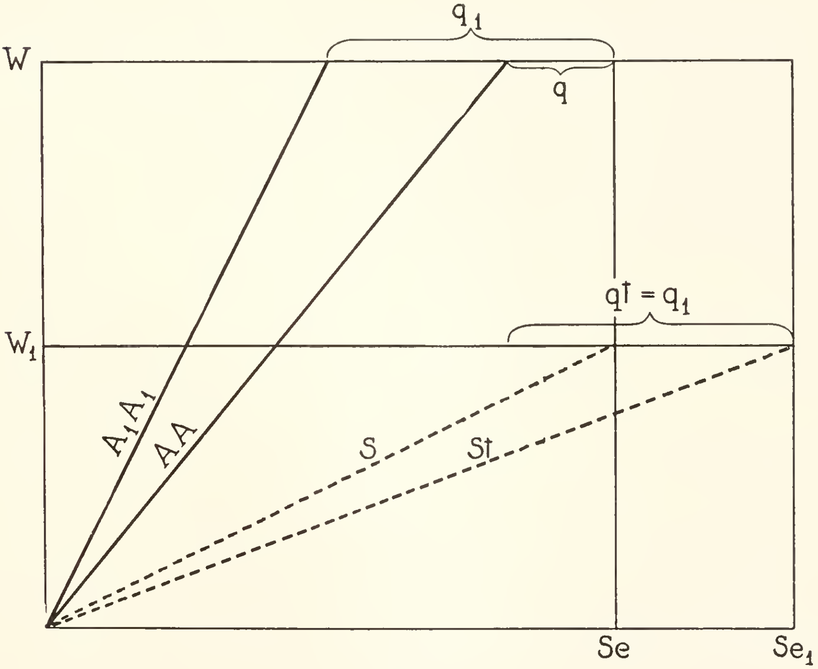

Fig. 4

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[18] Untersuchungen zur Entwicklungsphysiologie des Flügelmusters der Schmetterlinge. *Arch. Entw. mech.*, 47 (1920).

tive area is determined during the critical period which is supposed to end at the time *Se*. One of the simplest possibilities for the determination of the size of this area is, that it is proportional to the time which is available from the beginning of its formation to its final determination with the end of the critical period. Both of these points are, of course, determined independently and genetically, and we express this by assuming a genetic chain of reactions *AA* which reaches its active minimum at the level *W*, and a second chain *S* which determines similarly the time at which the critical period ends, *Se*. The distance between the two times, *q*, then is proportional to the area of the pattern-element in question. Now we might have another race in which genetically the curve *AA* is replaced by A_1 A_1, and therefore the area of the pattern in question is proportional to the distance q_1, that is, bigger. If I perform now a temperature experiment during the critical period, and the *S* and *A* chains have a different temperature-coefficient, I might shift *Se* to Se_1, without touching *AA*. Now our area is proportional to the distance *qt* which is equal to q_1, and the phenotype is exactly identical with the one of the race A_1. Speaking generally, we learn from this diagram that it may be possible within a system of timed reactions to produce a certain new phenotype by shifting one of the reactions, by changing its velocity. This shifting, however, and therefore the same effect, may be due either to an external agency like temperature, or to a mutation of the gene which lies at the basis of the reaction in question.

There is one consequence of these considerations which seems rather important. In such a system of timed reactions, there are not many degrees of freedom imaginable for the individual reaction, which would not upset the whole system. Therefore viable mutations are limited, and furthermore within such a system viable mutations are only imaginable, the phenotype of which might theoretically also be obtained by proper external action as modifications. This means that if we know the proper agents and the proper critical periods, we ought to be able to produce also the phenotype of every imaginable or known mutation in the form of a non-heritable modification. Putting aside the manifold obvious cases of this type in quantitative characters like size, I might mention that I succeeded in producing the exact phenotype of a considerable number of *Drosophila*-mutations as non-inheritable modifications through the action of extreme temperatures at different critical periods. It is very significant that in such experiments usually the modification in question appears simultaneously in a series of degrees, paralleling exactly series of known or also not yet known multiple allelomorphs.[19] If we remember what we heard before about such series and the reaction velocities, the wonderful consistency of all the facts and their connection through a rather simple idea becomes once more apparent.

I do not think that much imagination is needed to apply the different models of the argument, which have now been discussed, to any imaginable process of differentiation which proceeds orderly with time, and I believe that the relation between the gene and that part of the process of embryonic differentiation which belongs to the dimension of time is adequately explained by the system of timed reactions and what belongs to it. This, however, is only a part of the problem of embryonic determination. There is, in addition, the differentiation of the substratum in the three dimensions of space without which the reaction system which produces the right thing at the right time could not be imagined to produce it also in the right place. There can be no doubt that the spatial differentiation of the substratum is also produced at definite times by the same system of genic and timed reactions. Under normal conditions, a certain embryonic area, say a limb-bud, is equipotential up to one moment and differentiated into parts of different potency from that moment on. And this time of determination may be different but genetically fixed in nearly related species. We have discussed this point already in regard to the wing pattern. Further, all the elementary facts of experimental embryology, beginning with the analysis of the different types of eggs in regard to determination, prove that the progress of differentiation may be dissolved into a series of exactly timed events, consisting mainly in some diversification of the substratum, be this the egg which is to be regarded as an individual system, be it progressively smaller and smaller areas of the embryo, now to be regarded as the individual systems, which change at a certain moment from a monophasic to a polyphasic condition.[20] The causation of this change still belongs to the domain of physiological genetics, and is adequately understood by the system of timed reaction velocities. But in what this change consists and what are its consequences in regard to determination,—this is the proper domain of experimental embryology. The experimental facts have been described under many headings since the days when His first understood the problem with a really prophetic vision. Organ-forming stuffs, chemo-differentiation, embryonic segregation, and the organizator are all terms for the observed facts of the same order. The organizator conception, in addition, has led an important step further, because it connects the facts of the diversification of the substratum with former causative events and therefore opens the way for a dynamic understanding of a sequence of events, which has been started at one point. And the theory of the metabolic gradients, which constitutes the physiological corollary to the morphological organizator-concept, makes visible one of the ways for a causal explanation of the whole process.

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[19] Only a short notice has been published, though a considerable material has been accumulated. Jollos, who has repeated the experiments with the same results, is preparing a communication which relieves me from publishing the details of my results.

[20] Detailed discussion in "Physiologische Theorie der Vererbung."

Whatever this process of the diversification of the substratum, or in one word including all visible types, the process of stratification, might be, its meaning within the genetic system of timed reactions is clear. It allows the products of the genic reactions to act or not to act or to act differently on different areas of the germ, it creates secondary and tertiary systems, influencing the course of the genic reactions differently in the different regions, allowing one and the same original chain of reactions to lead to different consequences in the different areas, and the same over and over again up to the end of differentiation.

It would be pleasant to point to a few of the consequences which might be derived from such views as the ones presented here, consequences in regard to special and general problems of genetics, problems of mutation, evolution, the understanding of rudimentary organs or embryonic recapitulation. But these conclusions may be easily drawn by anybody who is willing to accept the soundness of the basic idea.[21]

Ladies and Gentlemen! A few years ago, one of the leading biologists of this country professed right here his opinion that the time has not yet come for genetics to join hands with experimental embryology. Permit me to conclude this lecture by expressing most emphatically my conviction that not only this time has long since come, but also that the foundations for an understanding of development from the standpoint of physiological genetics have already been laid. Indeed a considerable part of the frame-work stands ready around which to erect a good building.

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[21] Some of them have been presented in Die quantitative Grundlage etc., Materialien zur Theorie etc., and " Physiologische Theorie, etc." quoted before; others have meanwhile been drawn by other authors, who accepted the general trend of our ideas.

THE ORIGIN AND BEHAVIOUR OF CHIASMATA

V. Chorthippus elegans

C. D. DARLINGTON

John Innes Horticultural Institution, Merton, London

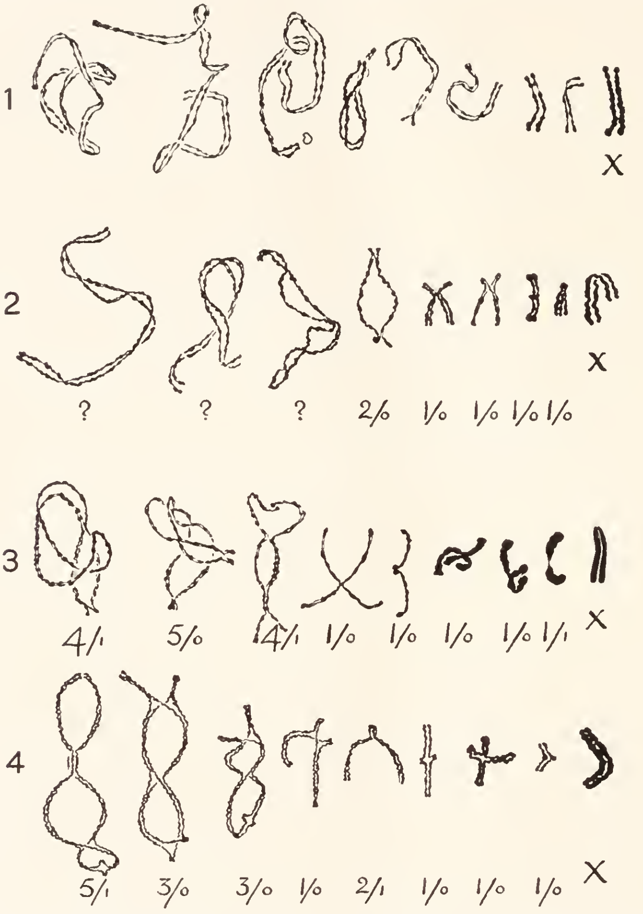
Introduction

At the pachytene stage of the prophase of meiosis the chromosomes are single threads and these threads lie side by side in pairs throughout their length: association is complete. But at the end of this stage the threads fall apart; they appear to repel one another and they are found to be longitudinally double, owing to the division of each chromosome into two "chromatids." The double threads remain associated, however, in spite of their repulsion, for the chromatids change partners at one or more places which are known as "chiasmata." The chiasmata persist with various changes in number and position from this diplotene stage until metaphase and they are important, first, on account of their function in holding the chromosomes together and allowing them to continue paired until metaphase, and, secondly, on account of their being the result of genetic crossing over between chromatids of opposite chromosomes (*cf.* Darlington, 1932). In order to understand their cause and function properly it is necessary to record their varying numbers and positions at successive stages of prophase in large numbers of organisms and so deduce their behaviour and the forces determining their origin and maintenance.

These forces have very different effects in different organisms and the subject of the present study, *Chorthippus elegans* (Acrididæ) is an example of a species with the least possible change in the number and position of the chiasmata between diplotene and metaphase. The methods used were the same as in *Stenobothrus* (Darlington and Dark, 1932) except that all the observations are from one smear preparation of one individual (giving the maximum uniformity of material). This was stained with Heidenhain's hæmatoxylin instead of gentian violet; the result, though less satisfactory for tracing the course of the separate chromatids at diplotene, is relatively free from danger of fading.

Observations of Spermatogenesis

Pachytene.—Eight double threads, the paired autosomes, can be distinguished together with one more condensed and homogeneous body, the X chromosome. No doubleness can be definitely made out in the autosome threads until the end of this stage, but the upturned ends of chromosomes show what may be the beginnings of a split. The X chromosome, however, has already divided into two halves. There is little evidence of a "bouquet" stage at zygotene and none persists through pachytene.



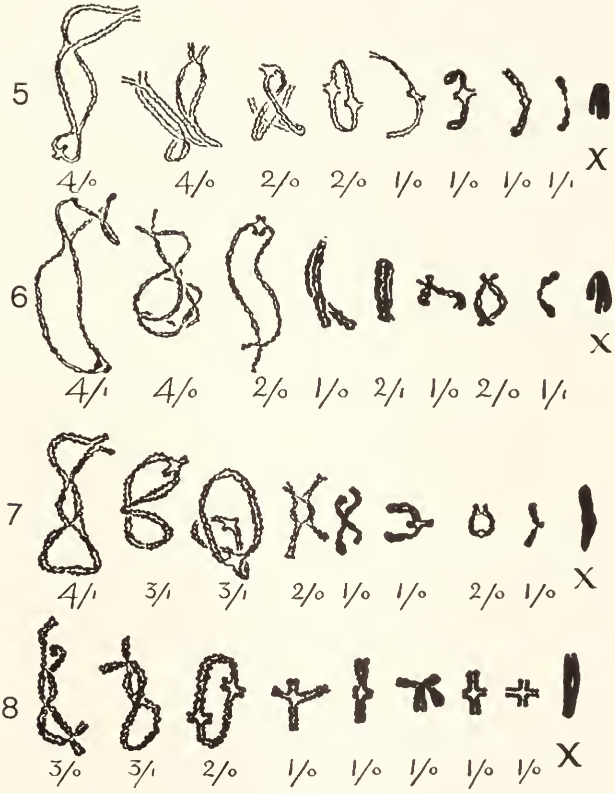
Figs. 1–12. The eight paired autosomes and the X chromosome at successive stages of prophase. The total number of chiasmata and the number terminal are given under each bivalent in the later stages. × ca. 2000.

Fig. 1. The end of pachytene.

Fig. 2. The beginning of diplotene. Separation of the shorter chromosomes complete.

Figs. 3 and 4. All loops completely open and chiasmata recognizable.

Diplotene to Diakinesis.—The paired threads fall apart (Fig. 1). The long chromosomes with median constrictions begin to separate either in the middle or at one end. Doubleness is still not evident.[1] The separation is first complete in the short chromosomes (Fig. 2). These are also found to be slightly in advance of the longer ones in condensation (Fig. 3) and they maintain this difference until diakinesis. This suggests that differential precocity ("heteropycnosis") here de-



FIGS. 5–8. Successive stages of diplotene.

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[1] Robertson and others have stated that the chromosomes are double before they pair but do not claim to have any direct evidence to show that this is so. The *indirect* evidence they bring forward consists in the well-known supposition of "anaphase duality" which is now seen to depend on a misinterpretation of spiral structure (Darlington, 1932). On the other hand, all *direct* observers of zygotene in plants and animals agree that the chromosomes are single at this stage (Gelei, Wenrich, Belling, Newton and Darlington). Speculations as to the possible earlier division of the chromosomes at meiosis and mitosis conflict with the precocity theory of meiosis (Darlington, 1931) but are not valid evidence against it.

pends on size differences. The loops first meet, revealing the number and position of the chiasmata, in the shorter pairs. As the loops open the doubleness of the chromosomes becomes detectable (Fig. 2). The chromatids can then be followed separately through the chiasmata and sometimes throughout their length. Later they swell considerably and cease to be separately identifiable, except at the chiasmata (Fig. 9). This swelling is seen in many animals (perhaps *Pristiurus* may be regarded as the extreme type) and also in a gymnosperm, *Taxus baccata* (Dark, unpublished). It is characteristically different from the behaviour in the angiosperms where the more swollen condition prevails throughout the post-pachytene stages and seems to prevent the chromatids being so clearly distinguished at diplotene as in some Orthoptera.

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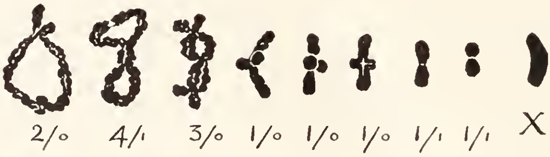

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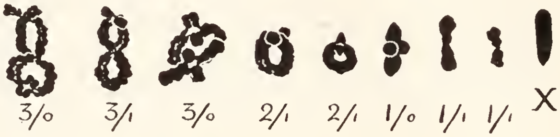

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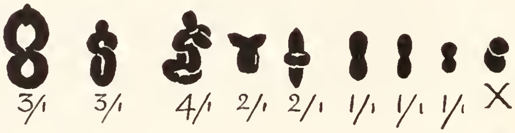

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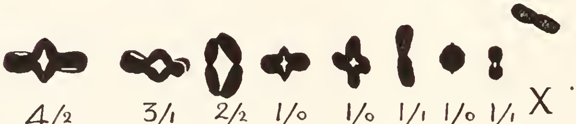


Figs. 9 and 10. Diakinesis.

Fig. 11. Pro-metaphase.

Fig. 12. Metaphase, X chromosome lying to one side of the spindle. *Note:* the three long pairs of chromosomes have median spindle attachments, the rest terminal.

The differences between angiosperm and orthopteran may, of course, be artefacts but it is impossible to say which is normal and which the artefact. Many would infer that the clearer observation is more true-to-life but such a conclusion does not necessarily follow. The increase in size has a curious effect. Owing to the differential but somewhat variable rate of condensation of short and long chromosomes, their relative sizes during the diplotene phase change rather suddenly and interfere with the constant distinction of types (Figs. 3–6).

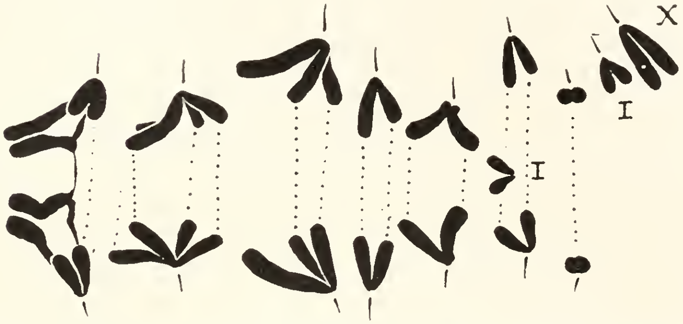
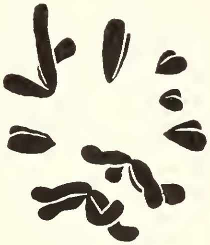
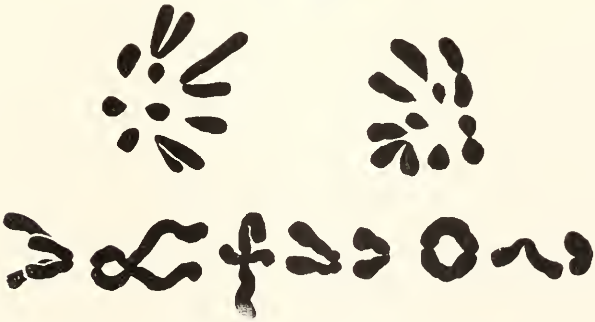


Fig. 13. Anaphase of the first division following failure of pairing of the shortest medium pair; one of the univalents is about to divide at the first division. The chromatids that have been associated are connected by a dotted line. × ca. 3000.





Figs. 14 and 15. Second division. × ca. 3000.
Fig. 14. Polar view of the eight autosomes in metaphase.
Fig. 15. The two divisions of one spermatocyte. Above, anaphase; nine chromosomes including the X chromosome. Below, metaphase (chromosomes drawn separately); the distal ends of some of the chromosomes are coming together.

Metaphase to Anaphase.—Between pachytene and metaphase the chromosomes contract to about one-sixth their length. The eight bivalents lie on the equatorial plate and the X chromosomes to one side of it (Fig. 12). Repelling one another from their spindle attachments the pairs of chromatids associated at these points pass to opposite poles. Those pairs distal to the first chiasma therefore have to separate and the strain often draws them into a fine thread. Exceptionally a connection is seen between the separating chromatids (Fig. 13), as already noticed in *Hyacinthus* and *Stenobothrus*. This connection is as yet unexplained. Exceptionally also two of the shorter chromosomes are found to be unpaired and may lag and divide at the first division. This failure corresponds with observations during prophase and, as will be seen, is related to conditions of chiasma formation.

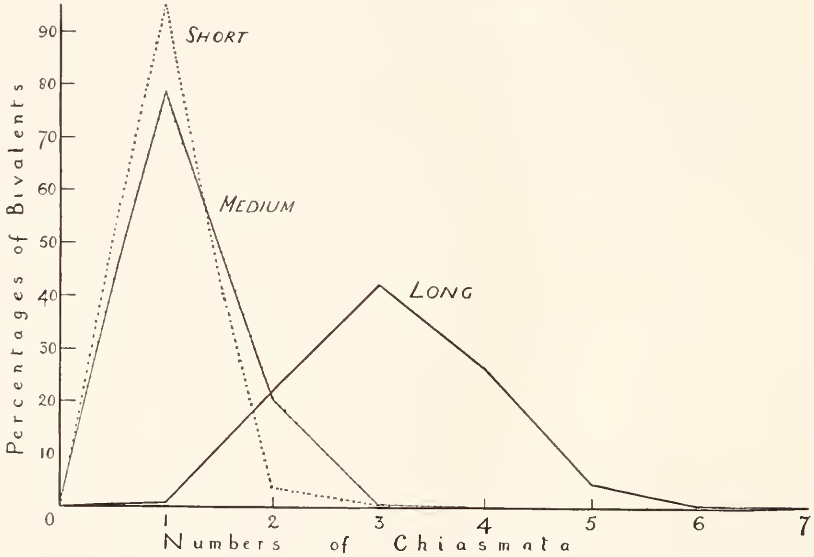


Fig. 16. Graph showing variation in chiasma frequency in the long, medium, and short types of chromosome.

Second Division.—During the interphase and early metaphase of the second division the pairs of chromatids are held together only at their spindle attachments (Fig. 14). They suddenly come together, immediately before anaphase, it must be presumed, for they are rarely seen together during metaphase and then only touching at the ends (Fig. 15).

Syndiploidy.—Groups of from two to six spermatocyte nuclei are often found closely appressed, between pachytene and diakinesis; and at metaphase, in a corresponding proportion of cells, fusion of the adjoining plates of chromosomes is seen. Where only two nuclei lie together the result is a regular fused spindle but, with a larger number, the chromosomes no longer orientate themselves regularly. These results are closely paralleled by Eisentraut's observations (1926) on *Gomphocerus maculatus* and by numerous observations of syndiploidy in plants, although the exact time of fusion, the onset of metaphase, is not elsewhere so clear. It is evident that in all these cases fusion takes place after pachytene and, although four homologous chromosomes of each type are present (in both plants and animals), quadrivalents are never formed. Evidently, therefore, the pairing of chromosomes at metaphase in these organisms can only be derived from pachytene pairing and does not arise from a direct affinity. This conclusion follows from the chiasma theory of pairing and contradicts the assumption that the chromosomes are paired at metaphase on account of any attraction operating between them at this stage (Darlington, 1931).

Table I

Summary of Observations of 100 Nuclei (800 Bivalents)

Numbers of Terminal Chiasmata in each Bivalent		Numbers of Chiasmata per Bivalent											
		long type (three)						medium type (four)				short type (one)	
		1	2	3	4	5	6	0	1	2	3	1	2
Mid Diplotene (24 nuclei)	0	—	11	20	9	4	—	—	67	11	—	8	—
	1	1	3	9	7	2	—	—	3	15	—	14	2
	2	—	1	2	1	1	1	—	—	—	—	—	—
Total		1	15	31	17	7	1	—	70	26	—	22	2
Late Diplotene (16 nuclei)	0	—	8	10	4	—	—	—	41	7	—	3	—
	1	—	3	13	7	—	—	—	10	6	—	13	—
	2	—	—	1	—	2	—	—	—	—	—	—	—
Total		—	11	24	11	2	—	—	51	13	—	16	—
Diakinesis (40 nuclei)	0	2	14	17	2	1	—	1	90	—	—	4	—
	1	—	12	30	15	2	—	—	43	25	1	35	1
	2	—	1	12	10	2	—	—	—	—	—	—	—
Total		2	27	59	27	5	—	1	133	25	1	39	1
Metaphase (20 nuclei)	0	—	3	3	1	—	—	—	31	—	—	4	—
	1	—	9	12	9	—	—	—	30	18	1	15	1
	2	—	1	8	14	—	—	—	—	—	—	—	—
Total		—	13	23	24	—	—	—	61	18	1	19	1
Grand Total..............		3	66	137	79	14	1	1	315	82	2	96	4
Percentages		1	22	45.7	26.3	4.7	0.3	0.3	78.7	20.5	0.5	96	4

Chiasma Frequency.—The numbers of chiasmata present in the bivalents of each type remain without significant change from diplotene to metaphase. They show (Fig. 16) the interference curves of frequency variation constantly observed in all organisms so far studied (Haldane, 1931; *cf.* Darlington, 1932). They also show (Table II) the indirect relationship of length of chromosome to frequency already found in *Stenobothrus* and probably very general in organisms with

TABLE II

Mean Chiasma Frequencies per Bivalent (derived from Table I)

		Mid Diplotene Stage (24 nuclei)	Late Diplotene Stage (16 nuclei)	Diakinesis (40 nuclei)	Metaphase (20 nuclei)	Total (100 nuclei)	Observations on Stenobothrus parallelus	Length
Long Type	No. Bivalents	72	48	120	60	300		11.3 μ
	Total No. Chiasmata	233	148	366	191	938		
	No. Terminal Chiasmata	34	29	109	76	—		
	Chiasma Frequency per Bivalent	3.24	3.08	3.05	3.18	3.13	3.31	
	Terminal Chiasmata per Bivalent	.47	.60	.91	1.27	—		
Medium Type	No. Bivalents	96	64	160	80	400		4.1 μ
	Total No. Chiasmata	122	77	186	100	485		
	No. Terminal Chiasmata	18	16	69	49	—		
	Chiasma Frequency per Bivalent	1.27	1.20	1.16	1.25	1.21	1.45	
	Terminal Chiasmata per Bivalent	.18	.25	.43	.61	—		
Short Type	No. Bivalents	24	16	40	20	100		1.6 μ
	Total No. Chiasmata	26	16	41	21	104		
	No. Terminal Chiasmata	16	13	36	16	—		
	Chiasma Frequency per Bivalent	1.08	1.00	1.02	1.05	1.04	1.04	
	Terminal Chiasmata per Bivalent	.61	.81	.90	.80	—		

wide range of size amongst the chromosomes (Darlington and Dark, 1932). This effect is not so pronounced, however, in the medium chromosomes as in *Stenobothrus* with the predictable result (on the chiasma theory of pairing) that the shortest member of this type occasionally fails to pair (Fig. 13, anaphase, and Table I, diakinesis).

Terminalization.—The total number of chiasmata remains the same, but the proportion of these that are terminal increases during prophase and it increases in a characteristically different way in the different types of bivalent and in those with different total numbers of chiasmata (Table III and Fig. 17). This agrees with the assumption that the terminal chiasmata arise by movement of earlier interstitial ones to the ends. Thus, amongst those with 2, 3, and 4 chiasmata, the proportion that are terminal (the *terminalization coefficient*) is the same at each stage. This indicates that originally all the chiasmata were interstitial as required by the hypothesis. Further, as in *Tulipa* (Darlington and Janaki Ammal, 1932), those with one chiasma show less movement, especially in the early stages, than those with two or more. Finally, the occurrence of more terminal chiasmata at the same stages in the corresponding classes of the shorter chromosomes is to be expected on the assumption of movement since the chiasmata are more concentrated in these chromosomes and have not so far to go to reach the ends.

TABLE III

Numbers of terminal chiasmata per bivalent in bivalents with different total numbers of chiasmata (derived from Table I)

Stage	Long Type			Medium Type		Short Type
	2	3	4	1	2	1
Mid Diplotene	.33	.42	.53	.04	.58	.64
Late Diplotene	.27	.62	.64	.20	.46	.81
Diakinesis	.52	.91	1.30	.48	1.00	.90
Metaphase	.84	1.22	1.54	.49	1.00	.79

The only change that takes place in terminalization in the long chromosomes in this organism is the expansion of closed loops at the expense of the distal arms, a change which according to the electrostatic hypothesis (Darlington and Dark, 1932) is due to a repulsion between distributed surface charges on the paired chromatids. In the medium and short chromosomes there is also a slight movement of single chiasmata owing to the special repulsion of the spindle attachments, but this movement is ineffective in moving a proximal chiasma against the repulsion of a loop when two are present owing to the terminal spindle attachments always lying in open arms in which repulsion is less effective.

The terminalization of the chiasmata in the short chromosomes is therefore very like that in the fragments of *Fritillaria imperialis* where all chiasmata are terminalized early although only the distal chiasmata move to the end in the major chromosomes with numerous chiasmata (Darlington, 1930).

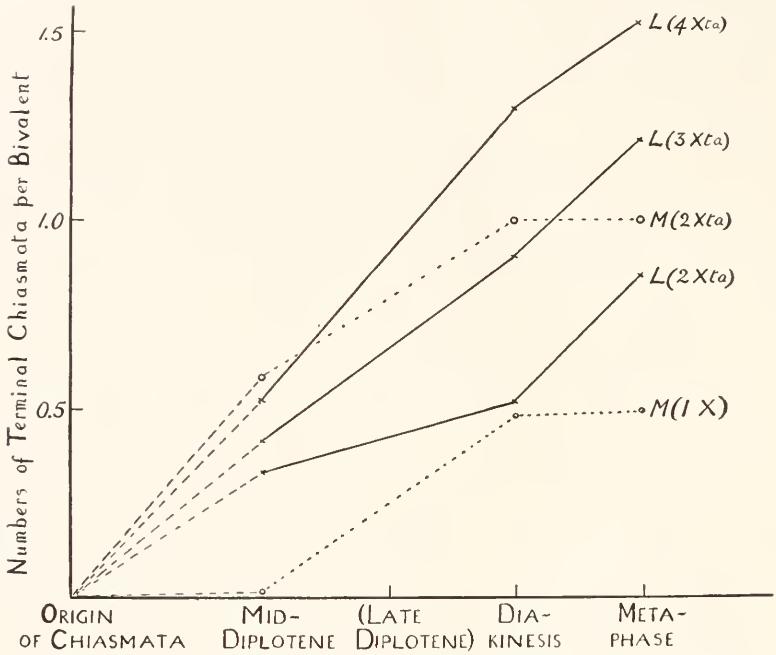


FIG. 17. Graph showing that the increase in the numbers of terminal chiasmata is in proportion to the total number at all stages where more than two are formed but not in the earlier stages where only one is formed. The short chromosome type and the late diplotene stage are omitted because the numbers of observations are smaller than those given.

SUMMARY

1. A study of meiosis in male *Chorthippus elegans,* Acrididæ ($2n = 16 + X$) shows the chromosome behaviour to be similar to that already described in *Stenobothrus parallelus.* Thus the chiasma frequency is an indirect function of length and has an interference curve of variation. Failure of pairing occurs in one chromosome type with a frequency in keeping with the curve. Terminalization depends entirely on the generalized repulsion in bivalents with closed loops and the localized spindle attachment repulsions are of the minimum degree found in *Fritillaria.*

2. A more extensive quantitative study makes it possible to show in the long chromosome type, that the number of terminal chiasmata at each stage between diplotene and metaphase is proportional to the number of interstitial chiasmata and increases from one stage to the next *pari passu* with the decrease in the number of the interstitial chiasmata. This confirms the earlier arguments that all interstitial chiasmata become terminal by movement without breakage while, on the other hand, terminal chiasmata always arise from earlier interstitial ones and in no other way. It is now clear that chiasmata always change their position after their formation at diplotene so that the configurations observed later are merely positions of changing equilibrium.

3. The opening of the diplotene loops has been followed in detail and shows that the chromosomes are single and undivided until this stage. The opening is therefore derived solely from the so-called " reductional " split, not from the " equational " one which only begins to appear at this time.

4. Syndiploidy occurs frequently just before metaphase.

For bibliography, see p. 370 of the following paper by the same author.

THE ORIGIN AND BEHAVIOUR OF CHIASMATA

VI. Hyacinthus amethystinus

C. D. DARLINGTON

John Innes Horticultural Institution, Merton, London

The original frequency and distribution and the later behaviour of chiasmata have been made clear in a number of organisms by earlier studies in this series and the comparison of observations on *Campanula, Tulipa, Fritillaria,* and *Stenobothrus* has shown us how to analyse the changes undergone by chiasmata in terminalization and has enabled us to define the forces at work in producing these changes (Darlington and Dark, 1932). Hence it is now possible to recognise from typical metaphase conditions of other organisms what the prophase conditions preceding and determining them must have been. This method can be satisfactorily applied to many species in which the prophase is not amenable to direct study.

A number of species of plants and animals are known with an extreme range in the sizes of their chromosomes (*cf.* Darlington, 1932). In species with slight size range the number of chiasmata formed is as a rule roughly proportionate to the lengths of the chromosomes at pachytene (*e.g.,* in *Hyacinthus orientalis, Fritillaria imperialis,* and *Vicia Faba*). This would only be possible with a great range of size if the longer chromosomes had a very high number, for the shortest must always form one chiasma, according to the chiasma theory of pairing, to ensure that they pair regularly (Darlington, 1930).

The indirect size-frequency relation expected on this theory has been found in *Stenobothrus* and *Chorthippus* where the extreme lengths are as 8 to 1. It may be inferred on the analogy of the observations in *Brachystola, Yucca flaccida* (O'Mara, 1931), and in the South American Acrididæ (Saez, 1930). In the last the abnormal frequency relation is evidently due to localization of chiasmata near the spindle attachment so that the same length of chromosome is concerned in forming chiasmata in all the chromosomes. In the other examples the distribution is even and the mechanism controlling the abnormal length-frequency relation cannot be so directly inferred.

High size ranges occur in many species of monocotyledons. In *Eucomis bicolor,* $2n = 32$ (Fig. 1) (*cf.* Müller, 1912), and in *Hyacin-*

thus amethystinus, $2n = 28$[1] (Fig. 2), the range is about 20 to 1, *i.e.*, the same as in *Drosophila melanogaster*. It is to be noted that the shortest chromosomes are too small to attain the characteristic chromatid breadth of the species. In *Hyacinthus* the complement consists of 10 chromosomes about 5 μ long together with 18 less than 1 μ long and an average about one-tenth the length of the longer chromosomes.

At the first metaphase of meiosis 14 bivalents are regularly found in *Hyacinthus amethystinus*. Polar views show the five long pairs with two or three chiasmata (Fig. 3). The detailed structure of the nine short pairs cannot be determined from this aspect. As always, it is necessary to examine bivalents of this size in side view. (This is not yet generally appreciated.) It is then found (Fig. 4) that most have a single terminal chiasma, a few have a single interstitial chiasma and occasionally there is one with two chiasmata. The mean chiasma frequencies of long and short types in this division are 2.4 and 1.1 respectively.

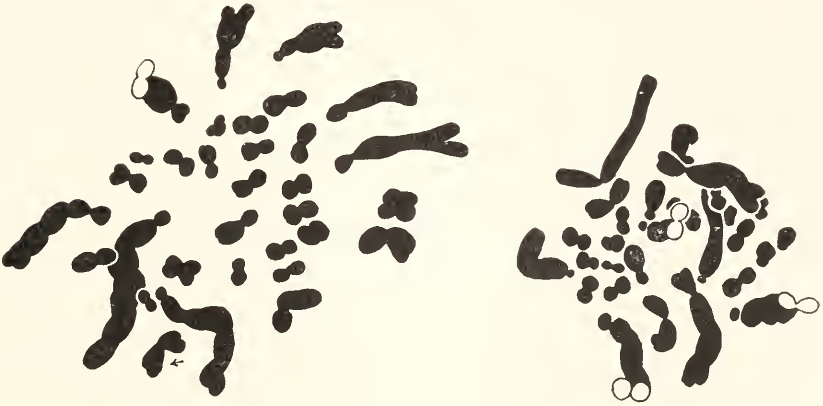


FIG. 1 FIG. 2

FIGS. 1 and 2. Mitotic metaphases from the root tip. × 3200.
FIG. 1. *Eucomis bicolor*, $2n = 32$.
FIG. 2. *Hyacinthus amethystinus*, $2n = 28$.

These observations show the closest analogy with *Stenobothrus* and *Chorthippus* except that the departure from normal in length-frequency relation is even more pronounced. The degree of terminalization is the same: the terminalization coefficient for the longer chromosomes is .45 in *Stenobothrus* and .42 in *Hyacinthus*, for the shorter chromosomes .67 in *Stenobothrus* and .60 in *Hyacinthus*. It is therefore evident that the conditions of terminalization are similar in the two instances and that since chiasma frequency is not reduced during prophase in *Stenobothrus* it is similarly unaltered in *Hyacinthus,* the only change being a movement of interstitial chiasmata to the ends of the chromosomes.

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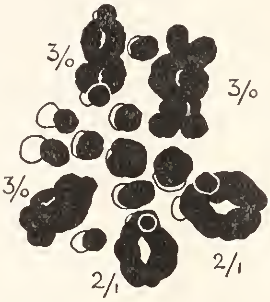
[1] The somatic chromosome number is given by Heitz (1926) as 24.



FIGS. 3 and 4. First metaphase of meiosis in *Hyacinthus amethystinus.* × 3200. The total numbers of chiasmata and numbers terminal are given. Sections cut at 24 μ.

FIG. 3. Polar view. The structure is only identifiable in the long bivalents.

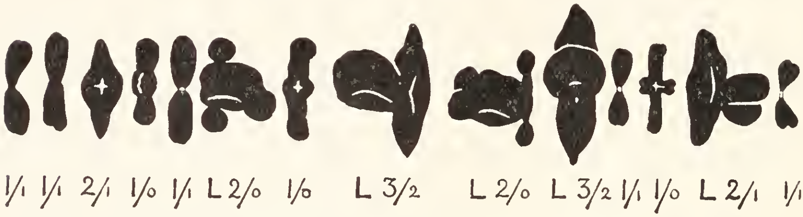


FIG. 4. Side view, bivalents drawn separately.

SUMMARY

The longer chromosomes of *Hyacinthus amethystinus* are on the average ten times the bulk of the shorter ones but have only twice as many chiasmata per bivalent. Thus, although the longer chromosomes form only two or three chiasmata, the shortest chromosomes regularly form one chiasma which ensures their regular pairing. This abnormality is characteristic of particular species and, like other variations in chiasma frequency and distribution, it must be genetically controlled. It is therefore to be regarded as an adaptive property.

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DOMINANCE OF TWO KIDNEY ALLELOMORPHS IN HABROBRACON JUGLANDIS (ASH.)

B. R. SPEICHER

(*From the Zoological Laboratory, University of Pittsburgh, and the Marine Biological Laboratory, Woods Hole, Massachusetts*)

Introduction

An allelomorphic series of at least four factors affecting the size of the compound eye has been located on the first chromosome of *Habrobracon*. The present paper deals with the effects of two of these. One, extreme small (k^e), was reported (Dunning, 1931) by W. F. Dunning and was kindly sent by her to P. W. Whiting. The eye size of mutant-type wasps showing this character is extremely variable, ranging from a total lack of eyes to those which, though approaching, never reach the normal size. The individual facets, when present, are of normal shape and size, the variation occurring as a decrease in the total number. The sizes of right and left eyes vary somewhat independently of each other. The ocelli are likewise affected and are also extremely variable, ranging from none at all to those apparently normal in size. Although no actual measurements have been made, it has been noted that the ocelli of any one individual tend to be of the same size. Aside from the modification of compound and simple eyes no other external effect is manifest. The mutant-type exhibits excellent viability when reared under standard conditions.

Kidney (k), an X-ray mutation found (Whiting, 1932) by Anna R. Whiting, presents superficially all of the characteristics of extreme small, but shows a high percentage of lethality when reared at standard temperature (30° C.). At lower temperatures these wasps show a viability equal to that of extreme small.

The striking similarity of the two mutants, coupled with their allelomorphism, suggested need for further study. Are they actually two separate factors having, except for the thermo-lethality of one, the same methods of expression, or is kidney a re-mutation to extreme small but carrying with it a closely linked factor causing lethality at higher temperatures? If the former hypothesis is correct, what are the conditions in regard to dominance? It was considered probable that a statistical study of the two types would bring out facts which, because of variability, would be obscure at casual inspection.

The writer is indebted to the Committee on Effects of Radiation on Living Organisms of the National Research Council for technical assistance through a grant to Dr. P. W. Whiting. Acknowledgment should also be made to Dr. Anna R. Whiting and to Dr. W. F. Dunning for making available the mutations here used, and to Dr. P. W. Whiting, at whose suggestion this work was done and whose help was greatly appreciated. The writer is likewise grateful to Miss Kathryn A. Gilmore for suggestions regarding technical difficulties.

Materials and Methods

Crosses Made

Two groups of females, one heterozygous for extreme small, the other heterozygous for kidney, were obtained by mating wild type (Stock No. 11) females to extreme small and to kidney males respectively. These heterozygous females were mated as follows:

(1) ♀ Kk^e by ♂ k^e.
(2) ♀ Kk^e by ♂ k.
(3) ♀ Kk by ♂ k^e.
(4) ♀ Kk by ♂ k.

The offspring from one half of the females of each of these four crosses were reared at 23° C. and those of the other half were reared at 30° C. All progeny were graded according to (1) the diameters of the compound eyes and (2) the size of the ocelli. Considering the number of mutant eyes counted, any measurement by facet count was impracticable. Consequently the lesser diameter of the eye was used as a basis of comparison.

Grading of Compound Eyes and Ocelli

Six grades of eye size were arbitrarily marked off (Fig. 1), of which four were set off in the following manner. From a stock of mutant-type wasps the individual possessing the largest eye was selected. With this eye as the upper limit of variability and the bare indication of the scleral ring surrounding the eye as the lower limit, four grades were established covering equal ranges in regard to eye diameter. These groups constituted Grades 1, 2, 3, and 4, in increasing order of size. During the process of grading the experimental material, a few eyes were found which exceeded the upper limits of Grade 4; these were placed in a separate group, Grade 5. Although, theoretically, Grade 5 extends from Grade 4 to wild-type, in no case did the sizes of any eyes belonging to this grade so closely approach that of type as to cause the two to be indistinguishable. It was considered advantageous to record separately the eyes which had failed to develop;

this group was designated Grade 0. Thus Grades 0 and 5 mark the extremes in variation of eye size with Grades 1, 2, 3, and 4 representing the intermediate stages.

Eyes were selected (to be used as standards of comparison with the experimental material) which met the theoretical specifications of the grades. As an aid to more accurate grading, each standard was made to represent the upper limit of its grade. Eyes under question as to their proper position in the series could then be compared with the standard most nearly resembling them and if of the same size or slightly smaller would be placed in the same grade as represented by the standard, but if they were larger they would be classified as belonging to the next higher grade. Each wasp was compared directly with these standards, the sizes of right and left eyes being recorded separately.

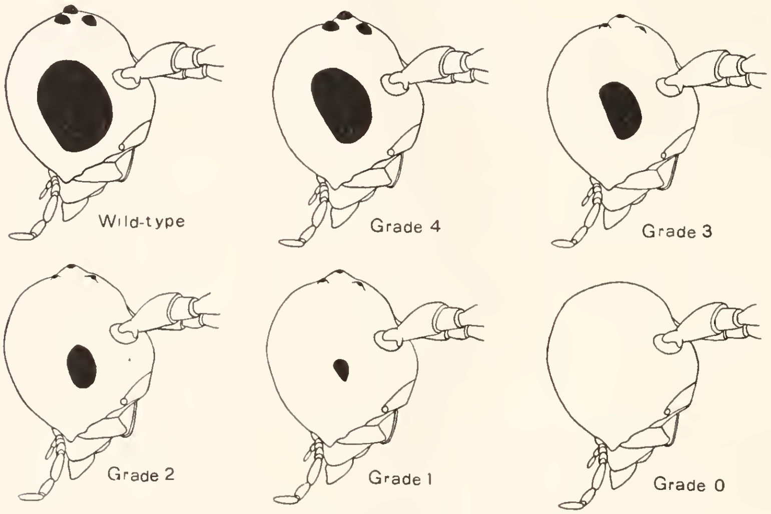

FIG. 1. Eyes of wild and mutant-type wasps. Illustrations of mutant-type wasps represent the upper limits of their designated grades. × 33.

The ocelli were divided into three groups, absent, small, and normal. Although, as mentioned above, the ocelli of any one individual tended to be of the same size, in case of disparity the wasp was classified in the small ocelli group.

Recording of Lethal Wasps

In *Habrobracon* lethal individuals may be recognized by the presence of shriveled larvæ or blackened pupæ still confined within the cocoons. While lethality occurring during the development of the egg may escape detection, some estimation of the number of lethal wasps may be made by scrutinizing the culture vials. Lethal counts given in this paper were made by using the above method.

PRESENTATION OF DATA

Comparative Lethality of Mutant Types

Among the four classes of wasps which were reared at 30°, two had an excessive number of lethal individuals. As kidney is known to be lethal when reared at this temperature, the fraternities containing kidney wasps may be expected to be the ones most affected. As seen in Table I, offspring of the crosses $Kk \times k^e$ and $Kk \times k$ contain by far the greater number of lethal wasps. The cross $Kk \times k^e$ produces kidney males and the cross $Kk \times k$ produces both kidney males and kidney females, thus accounting for the greater number of lethal individuals among the progeny of the former cross and the still greater number among those of the latter cross.

TABLE I

Numbers of type, mutant, and lethal wasps resulting from different crosses reared at different temperatures.

Origin	Wasps reared at 30° C.				Wasps reared at 23° C.			
	$Kk^e \times k^e$	$Kk^e \times k$	$Kk \times k^e$	$Kk \times k$	$Kk^e \times k^e$	$Kk^e \times k$	$Kk \times k^e$	$Kk \times k$
Type	301	432	344	198	222	275	239	182
Mutant	300	383	172	23	220	232	240	178
Lethal	71	44	119	134	47	54	33	45

The number of males produced in proportion to the females is an important factor affecting the comparative lethal ratios. An excess of males in a class coming from the cross $Kk \times k^e$ would raise the entire lethal ratio and, since the males of the lethal group were not separated from the females, the cause for the increase would not readily be apparent. In this case the sex ratio of the mutant-type must be considered, and may be assumed to be approximately the same as that of the type wasps of the same group. In the cross $Kk \times k^e$, reared at 30°, there was an excess of males, but not of statistical significance. In the other groups reared at 30° and 23° the sex ratios were likewise not significantly different.

Offspring from the cross $Kk^e \times k$ had a low lethal ratio. The mutant-type females of this group, being extreme small-kidney compounds, should demonstrate the dominance of one or the other factor in regard to thermo-lethality. The low lethal ratio and the abundance of viable compound females (Table I) indicate that the thermo-lethal trait associated with kidney is recessive. The lethal ratios of the four classes reared at 23° were not significantly different, since neither mutant factor causes an excess of lethals at this temperature.

A peculiarity brought out in Table I which is worthy of passing notice is the excessive number of mutant-type wasps in classes reared at both temperatures. Since the number of lethals counted represents the minimum actually produced, and the major part of the lethal group consists of mutant-type wasps, this excess is obvious, although the cause is as yet unexplained.

TABLE II

Grades of eyes and of ocelli tabulated according to genetic constitution and temperature. As regards eye size, the numbers in the body of the table indicate numbers of eyes graded. As regards grades of ocelli, the numbers indicate individuals.

	Wasps reared at 30° C.					Wasps reared at 23° C.				
	k^e	k^ek^e	k^ek	kk	k	k^e	k^ek^e	k^ek	kk	k
Eye size										
Grade 0	86	97	305	7	33	6	39			
1	83	51	120	4	11	19	38			
2	300	119	308	11	8	109	113	2	1	
3	115	23	55		4	84	71	41	6	6
4	14	2				56	11	624	193	308
5								3	2	6
Mean	1.81	1.25	1.14	0.68	0.64	2.60	1.92	3.94	3.97	4.00
Ocelli										
Lacking	1	4	19	10	13		2			
Small	289	141	374	1	15	136	134	320	10	5
Normal	9	1	1			1		15	91	156

Comparative Eye Sizes of Mutant Types

The effect of a 30° temperature on the eye sizes of the various genetic classes is seen in Table II. Most of the offspring from each of the four types of matings possess eyes which fall into the lower groups, Grades 2 and 0 being particularly well represented. The fact is well demonstrated in the classes unaffected by thermo-lethality and is suggested in the kidney classes, although in these latter groups the numbers are not sufficient to be statistically significant.

The dispersal of wasps according to the sizes of both right and left eyes is seen in Table III. Among the wasps reared at 30° the groups composed of extreme small females, compound extreme small-kidney females, and to a lesser extent the extreme small males show a preponderance of individuals in four places, involving Grades 0 and 2.

TABLE III

Correlation tables showing the relation of size of right and left eyes in wasps of different genetic constitution reared at different temperatures. Ordinates represent grades of left eyes and abscissæ represent grades of right eyes. The numbers in the body of the table indicate the number of individuals reared, the eyes of which fall in the designated grades.

		30°						23°					
		0	1	2	3	4	5	0	1	2	3	4	5
♂, k^e	5												
	4			7	1	1				5	10	17	
	3	5	7	24	19				1	12	17	3	
	2	12	15	84	35	1		3	5	29	22	3	
	1	11	9	22	2	2			3	2	4	1	
	0	16	5	16	4	1							
♀, $k^e k^e$	5												
	4		1					2	1	1		1	
	3	4	3	4	2				8	16	13	2	
	2	22	13	27	3	1		5	4	28	13	2	
	1	13	5	6	3			2	2	12	2		
	0	19	3	16	1			8	5	4	4	1	
♀, $k^e k$	5											2	
	4									1	10	296	1
	3	5	4	15	6						7	17	
	2	50	18	78	5							1	
	1	18	14	19	5								
	0	78	28	45	6								
♀, kk	5												1
	4										1	95	
	3										2	1	
	2	3	1	3								1	
	1	1		1									
	0	1	1										
♂, k	5												1
	4										1	151	3
	3				2						1	3	
	2	3	0	1									
	1	4	2										
	0	1	3	1									

Thus in the compound extreme small-kidney group in Table III, the areas 0, 0 (78 individuals), 0, 2 (50 individuals), 2, 0 (45 individuals), and 2, 2 (78 individuals) possess an abundance of wasps as compared with the surrounding squares. This indicates a tendency for the wasps to have the eyes either entirely lacking or developed up to a stage represented by Grade 2, which is apparently somewhat of a limit for eye size of wasps reared at this temperature.

The bimodality of each of these classes, as represented in Tables II and III, may be due in part to the method of measurement. The anatomical variation of the eye, although used as a basis of comparison, is a secondary result of a more fundamental but unmeasured physiological change within the organism. In Grade 0 this variation reaches a lower limit of expression. The physiological variation, however, does not stop here but grades down to the lethal point. Below the threshold of viability the effects are again perceptible externally, this time as lethals, the majority of which have the eyes entirely lacking and are noticeably micro-cephalic. There is, then, a range of physiological variation which can be seen externally only by Grade 0 and the lethal group, and as these two groups must contain those individuals falling into the intermediate physiological grades their numbers are thus increased. From this it appears that Grade 0 is not a true mode of eye size, the strong representation of individuals in this grade being a result of the methods used in calibrating the variation. The true mode is confined to the upper limit of variation, which for those classes reared at 30° centers around Grade 2. This explanation, although difficult to prove, is supported by the fact that in the arrays of eye sizes of wasps reared at 23° (in which cases the ranges of physiological variation have been raised) there is but one mode present, centering around the upper limits of variation.

A difference in the expression of the mutant factors appears in the classes reared at 23°. As seen in Tables II and III, extreme small wasps possess eyes which cover the same grades as do those of the corresponding group reared at 30°, although their means (Table II) are slightly higher. In contrast to this is the array of kidney wasps which at 23° have the eyes concentrated around a single high grade. The compound extreme small-kidney females follow the kidney trait in the concentration of eye size around Grade 4, indicating a complete dominance of kidney over extreme small in regard to this character.

A comparison of means of eye size is given in Table II. The difference between the means of extreme small and kidney wasps, both reared at 30°, suggests a difference caused by the two factors at this temperature, although the numbers are too small to be statistically significant.

In judging the variation of the ocelli of wasps reared at 30° the modes of the classes containing the factor extreme small are in the "small" group, as represented in Table II. In the kidney wasps the mode appears to have shifted down towards the "lacking" group, although here again the small number of viable wasps is not sufficient to be of any statistical significance. In the 23° groups, however, the numbers are large enough to make comparisons. At this temperature the extreme small groups retain their small ocelli, but in the kidney groups the majority of ocelli appear normal in size. The compound extreme small-kidney females resemble the extreme small group at this temperature in regard to the size of the ocelli.

Correlation in Size of Right and Left Eyes

At 23° kidney and kidney-extreme small compounds showed little variation in regard to eye size, and at 30° kidney wasps were too few to give significant results. Correlation coefficients were calculated as follows:

Extreme small males, reared at 30°, $r = 0.2939 \pm 0.0352$.
Extreme small females, reared at 30°, $r = 0.1129 \pm 0.0568$.
Extreme small-kidney females reared at 30°, $r = 0.2375 \pm 0.0353$.
Extreme small males reared at 23°, $r = 0.4520 \pm 0.0458$.
Extreme small females reared at 23°, $r = 0.2351 \pm 0.0546$.

It is evident that correlation in eye size exists although it appears to be not very high under the conditions of measurement.

SUMMARY AND CONCLUSIONS

1. Two mutant allelomorphs in *Habrobracon*, extreme small, k^e, and kidney, k, superficially resembling each other in their effects on eye size, were studied to determine their interrelationships.

2. Kidney wasps reared at 30° are for the most lethal, appearing in general as eyeless small-headed pupæ. Extreme small are viable at 30° and no lethal effect is evident at 23° in either type. Extreme small is dominant as regards thermo-lethality since extreme small-kidney compound females are of normal viability when reared at 30°.

3. In extreme small wasps size of compound eye is relatively little affected by temperature. In kidney, however, a lower temperature causes a much larger size. Kidney is dominant in this respect in the compound females.

4. In extreme small wasps size of ocelli is likewise relatively little affected by temperature, being "small" both at 30° and at 23°. In kidney, however, the majority reared at 23° have normal ocelli, while

at 30° ocelli are smaller or altogether lacking. Extreme small is dominant over kidney as to ocellar size both at 30° and at 23°.

5. There is a decided tendency in both mutant types for the compound eye either to be entirely absent or to develop to a certain modal size determined by temperature and genetic constitution.

6. There is a low but significant correlation as to size between right and left eyes.

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THE MOSAIC DEVELOPMENT OF THE ASCIDIAN EGG

N. J. BERRILL

Department of Zoölogy, McGill University, Montreal

The development of ascidian eggs has long been considered, as the result of the classical investigations by Conklin, to be the perfect example of the mosaic rather than the regulative type.

Different blastomeres of dividing eggs were killed or sufficiently injured to inhibit further development and the development of the remaining blastomere or blastomeres was followed. He found that each blastomere developed only into the tissues and organs that it would have produced had it remained a part of intact egg or embryo. In no case did a whole but dwarf larva result from one of the first two or four cells. The left blastomere of the 2-cell stage gave rise to the organs of the left side only. The anterior two blastomeres of the 4-cell stage gave rise to epidermal, neural, and chordal tissues, but failed either to gastrulate or to form a tail.

In general the organization of amphibian eggs is similar to that of ascidian eggs, formative substances appearing rather later, although Hall has recently shown that they are delimited as early as the 8-cell stage. Isolated blastomeres of the 2-cell stage, however, may give rise either to a whole larva of half the normal size or to a right or left half-larva of normal size, according to whether or not there has been reorganization of the cell contents.

In consequence of this it has seemed possible that the absence of regulation or reorganization in Conklin's experiments may have been due to the fact that the injured blastomeres were left in situ within the egg membrane. The presence of their inert mass in contact with the surviving part may have had an inhibitory effect. This possibility has been emphasized by Reverberi's conclusions, based upon experiments with *Ciona* eggs, that the eggs of ascidians should be placed properly within the regulative class.

The great obstacle to experimental work with ascidian eggs has been the difficulty of removing the egg membranes and follicle cells without injuring the ovum. It is partly the object of this paper to record a method by which this may be done. Several ascidian eggs are shown in Fig. 1, and it is seen that there is considerable variation in the character of these membranes.

It has already been shown (Berrill, 1929) that the membranes are normally digested from within by a proteolytic enzyme secreted by the developing tadpole, that the enzyme is fairly stable and is active within the limits pH 7.0–10.0. Trypsin digests the membranes, but only very slowly, so that the protease concerned is probably a tryptic ferment of a less specialized nature than trypsin itself.

There are, accordingly, two means by which membranes of ascidian eggs may be removed. The enzyme produced by developing ascidian tadpoles may be collected and concentrated, or a suitable and more readily obtainable substitute may be found. It was discovered that *Ciona*, *Phallusia*, *Ascidia*, and *Ascidiella* eggs remained about 100 per cent viable at 18° C. for a little over twenty-four hours, so that any enzyme mixture to be of use must be effective within that period.

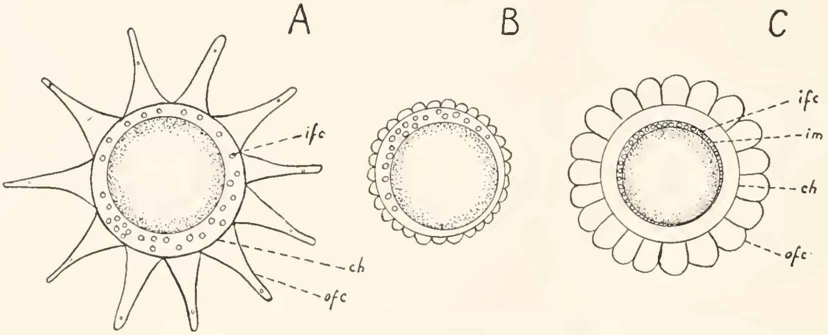

FIG. 1. Ascidian eggs. *A*, *Ciona intestinalis; B*, *Molgula* or *Polycarpa; C*, *Ascidiella aspersa*. *ifc*, inner follicle cell; *im*, inner membrane; *ch*, chorion or egg-membrane; *ofc*, outer follicle cell.

The natural ascidian enzyme in sufficient concentration is preferable to any substitute, but it can be obtained only from species of the family Ascidiidae (i.e., the genera *Ascidia*, *Ascidiella*, *Phallusia*) and *Ciona*. To obtain it in effective concentration it is necessary to make mass cultures of some thousands of embryos. Normal development of large numbers of eggs necessitates the use of a relatively large volume of water, the larger the better, for it is only when development has proceeded to within two or three hours of hatching that concentration becomes effective. Then the embryos are allowed to settle on the bottom of the vessels and as much supernatant water as possible is poured off. The remainder, together with the embryos, is poured into a test tube or cylinder, the embryos again allowed to settle, and the water reduced to about 5 cc. This with the embryos is then spread over the bottom of a Petri dish to ensure a supply of oxygen to the embryos, left there until hatching is complete, and the water then collected. It should contain enough enzyme to digest off the membranes from unfertilized *Ciona* or *Ascidia* eggs in from five to ten hours. Such eggs may subsequently be fertilized.

If it is desired to remove the membranes from merely a small number of eggs, the eggs may be placed among the developing tadpoles about three hours before they are due to hatch, when their membranes will dissolve about an hour after hatching (the developing tadpoles should be close enough together to be almost touching and to form a sheet one layer deep). It may be desirable to remove the layer of outer follicle cells by washing the eggs in a bag of fine bolting silk, when the enzyme can more readily attack the underlying membrane.

Large *Ascidia* or *Ciona* are not, however, always obtainable, and it is often more convenient to use a substitute for the ascidian enzyme. Such substitutes may be obtained from any large carnivorous or omnivorous invertebrate, the most active mixtures being the stomach juices themselves rather than gland extracts or preparations.

Those actually used were the clear juices found in the stomach of decapod crustacea such as *Munida*, *Maia*, and *Homarus*. No doubt other forms would be equally effective.

Undiluted, such juices are too potent and toxic, but a dilution of one part juice with fifty or one hundred parts of sea-water is both safe and efficient. The time taken for the membranes to be removed depends not only upon the activity of the enzyme but also on the thickness of the membrane and on the temperature. At 18° C. the membranes of *Ciona*, *Molgula*, or *Ascidia* are removed in from two to four hours, but the thicker membranes of *Polycarpa* or *Styela* require more than twenty-four hours. Unfertilized eggs of *Ciona*, *Molgula*, and *Ascidia* that have had their membranes removed in this way may subsequently be fertilized and undergo normal development.

If fertilized eggs are subjected to such a mixture or if eggs are fertilized while yet within it, a somewhat unexpected feature appears. Nuclear division proceeds normally, at least to the 32-cell stage, but cytoplasmic division is inhibited. In the case of later embryos, the surface cells round off and tend to fall away. These phenomena are apparently due to surface action of the lipochrome contained in the original digestion mixture, and in consequence this method of removing the membranes is satisfactory only in the case of unfertilized eggs. In this last case the capacity for fertilization and normal development is unaffected.

Once membranes have been removed, experiments of two kinds become possible. The naked eggs may be cut with glass needles, as used by Horstädius (1928), while blastomeres may be separated from one another. Attempts to cut the eggs were made only in the cases of *Ascidia* and *Ciona*. Those of *Ascidia* (*Ascidiella aspersa*) possessed a tough surface and a very fluid endoplasm, so that all attempts at cutting resulted in bursting them. The eggs of *Ciona*, on the other hand, are readily cut but possess such a viscous endoplasm that the injured surface does not re-form. In neither case, therefore, is experimental work of this nature feasible. It is just possible, however, that other eggs, such as those of *Molgula* or of other species of *Ascidia*, may be more suitable and of a type intermediate between those of *Ciona* and *Ascidiella*.

Separation of blastomeres, on the other hand, is very easy to effect. They may be separated with a glass needle, as used by Horstädius, or more simply merely by slight shaking. Pouring water in which there may be some hundreds of naked eggs in the 2-cell or in the 4-cell stage from one vessel to another several times results usually in the complete separation from one another of all blastomeres. There is a tendency among the blastomeres of *Ciona* eggs to fall apart in any case, though eggs and early embryos of *Ascidiella* tend to fuse on contact.

In the subsequent development of blastomeres isolated in the above manner no indication was found of any reorganization. Blastomeres isolated in the 2-cell stage invariably give rise to lateral half-embryos that gastrulate imperfectly and have but twenty instead of forty notochordal cells which, moreover, fail to interdigitate. Anterior blastomeres isolated at the 4-cell stage formed small spheres of ectoderm with about ten notochord cells loosely connected with the outer surface. Posterior blastomeres derived from the same stage formed small spheres of endoderm and mesoderm enclosed within ectodermal cells. Illustrations of such partial development are shown in Fig. 2. Each isolated blastomere apparently divides to form just those cells and tissues it would have formed had it remained a part of a whole embryo. Never was there any sign of development to form a whole larva of half or quarter normal size.

It is concluded, therefore, that Conklin's results are in no way invalidated by his failure to remove the dead or injured blastomeres or the egg membrane, and his conclusions concerning the mosaic nature of the ascidian egg are confirmed.

Reverberi, on the other hand, has reached conclusions of a very different nature. By an ingenious method of puncturing the chorion and compressing the ovum he succeeded in dividing *Ciona* eggs into two unequal parts, both parts subsequently being fertilized. His main conclusion, that the ascidian egg should definitely be assigned to the regulative class, is based upon two discoveries. The segmentation of the small extruded part of the ovum not only commences in a plane at various angles to the first cleavage plane of the ovum proper, but proceeds in a fairly typical manner; while eggs part of which have been extruded are often able to develop to form an apparently perfect tadpole larva.

These statements are not questioned, but they are by no means incompatible with the mosaic conception.

The development of the small extruded part is normal only in so far as the first three cleavages are in the three planes of space as in the normal egg. Gastrulation and coördinated development do not occur. Since, with the exception of the Nemathelminthes, the segmentation of almost all animal eggs is uniform, the first cleavage being vertical, the second vertical at right angles to the first, and the third horizontal, the ability of part of an ascidian egg to follow this same course is alone hardly evidence of strict regulation and any other sequence would be startling. The outstanding exception to such a course, namely the segmentation of the egg of *Ascaris*, results in a very unstable configuration. The spindle tends to lie along the longest cytoplasmic axis, and the fact that a fertilized part of an ascidian egg obeys this rule of Hertwig in the first stages merely emphasizes its validity. It has no real bearing upon the mosaic or regulative nature of the egg.

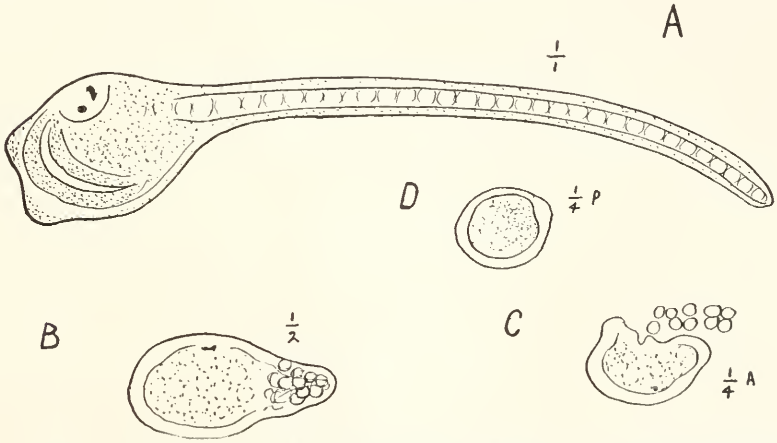

FIG. 2. Development of *Ascidiella aspersa* (24 hours at 17° C.). *A*, normal tadpole; *B*, development of isolated blastomere of 2-cell stage; *C*, of anterior blastomere of 4-cell stage; *D*, of posterior blastomere of 4-cell stage.

The other experiments supposedly showing a regulative capacity on the part of the egg concerned the development of diploid eggs, part of which had been extruded after fertilization. These developed to form apparently normal tadpoles of reduced size. In no case was it found possible to orientate the eggs with regard to their oöplasmic contents. The only illustrations, however, show two larvæ with tails about four-fifths full length and with a trunk region from about four-fifths normal to full size. The one with the smaller trunk has the longer tail, and if the part extruded consisted of part of the endodermal region of the ovum, the peculiarity of this tadpole is accounted for. Similarly, the large-bodied, short-tailed larva is explained if the part extruded contained more of the chordal crescent and less of the endodermal oöplasmic region. No growth in size, other than the swelling of notochord cells, occurs in *Ciona* until after the tadpole stage has been passed, so that the size of the tadpole is a fairly accurate indication of the size of the egg, and it is evident in these two cases that only a very small part can have been extruded. In any case the peculiarities of the two examples illustrated can be as readily explained on the basis of the mosaic conception as on the assumption that the egg is more regulative than is generally believed.

SUMMARY

A new method of removing the membranes from ascidian eggs in the mass is described. Essentially the method is the use of proteolytic enzymes.

The development of isolated blastomeres is described, confirming Conklin's results that the development of each is strictly partial and that the whole development is a mosaic. It is also shown that the presence of the egg membrane and of injured or dead blastomeres in his experiments in no way invalidated those results.

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THE EARLY EMBRYOLOGY OF THE ECHIUROID, URECHIS

W. W. NEWBY

Department of Zoölogy, University of Utah

Introduction

The eggs of *Urechis* are being used for many investigations in experimental embryology and a need for a study of the normal development has been felt. The object of the present investigation has been: first, to develop a technique whereby either normal or experimentally treated eggs may be stained and preserved for permanent record; second, to describe and figure the early stages of the development; and third, to compare the development of *Urechis* with that of *Thalassema mellita* (Conn), as described by Torrey, 1903.

This paper is a preliminary report, preparations for the further study of the development of the worm having been made. The work was begun in the summer of 1931, at the Hopkins Marine Station at Pacific Grove, California. During the following winter it was continued in the Zoölogical Laboratory of the University of Utah. It is with pleasure that the writer expresses his appreciation for the aid given him by Professor G. E. MacGinitie, of Hopkins Marine Station, in the collecting of the worms and the rearing of the larvæ, and his indebtedness to Dr. Harold Heath, under whose direction the work was carried on.

Material and Methods

The worm *Urechis caupo* was described by Fisher and MacGinitie in 1928. It is an inhabitant of the mud flats of the bays and sloughs along the California coast and dwells in U-shaped, tubular burrows. The worms used in this work were obtained from Elkhorn Slough at Monterey Bay and from Morro Bay. They were brought to the laboratory in glass jars cooled by ice packs and in the laboratory were kept in well-aërated aquaria.

The sexes are separate in *Urechis*, the eggs and sperm being stored in the six segmental organs, from which they may be easily obtained by gently probing into the ducts of the organs with a thin, smooth, glass probe. The eggs were placed in finger bowls of fresh, filtered sea water with the water about an inch deep. Enough eggs were placed in each bowl to cover the bottom with a single layer. The sperm placed in a watch-glass made a rather thick fluid. This was diluted with about ten volumes of sea water. Insemination was effected by the addition of three drops of the sperm dilution to each bowl of eggs. This produced almost 100 per cent fertilization with only an occasional case of polyspermy. The developing eggs were kept in a poorly lighted basement room where the temperature was nearly constant at about 20° C. At this temperature the rate of development was very constant for all the eggs, being remarkably uniform after the 16-cell stage.

The formation of the fertilization membrane, the polar bodies, and the first cleavage could be readily observed and samples of the eggs were fixed at each of these stages. After the 2-cell stage samples of the eggs were fixed every fifteen minutes.

The eggs were removed with about 2 cc. of the sea water and placed in a vial. They were fixed by the addition, drop by drop, of an equal amount of corrosive sublimate and acetic acid fixative (Guyer No. 18). After ten minutes the fluid was removed with a pipette and the eggs were washed with distilled water for two minutes. This was removed and washing was continued in 50 per cent and then in 70 per cent alcohol for two days. This treatment resulted in a shrinkage of less than 12 per cent of the diameter of the egg.

The eggs were stained for one hour and forty minutes in Mallory's phosphotungstic acid hematoxylin (Guyer No. 62). The stain was diluted to about 20 per cent and when used at this concentration, spindles of dividing cells, chromosomes, and cell boundaries were distinct. Little destaining was needed with this stain but when necessary it was done in a weak alkaline alcohol, the treatment being continued until the eggs were a light pink when observed with the low power of the microscope and with reflected light. The eggs were then dehydrated, cleared, and mounted in balsam. In all processes after the washing the eggs were not handled, but were placed in a small dish and all fluids were added or withdrawn with a pipette. The balsam was added after most of the xylene had been removed and small drops of this balsam with the eggs were easily mounted.

Contrary to a common opinion, the technique of staining and mounting is not laborious and the writer suggests that often much might be learned if experimentally treated eggs were thus preserved for closer examination.

Fertilization and the First Cleavage

The process of fertilization has been described by Tyler (1931). The process may be outlined as follows: first, the attachment of the sperm; second, the appearance of a cone on the surface of the egg immediately below the point of attachment; third, the fertilization membrane appears on the surface of the egg and begins to lift; fourth, the sperm entrance cone of the egg begins to enlarge and the indenture of the egg begins to round out; fifth, the sperm head enters the egg. This process takes six and a half or seven minutes at 20° C. At a time about twelve minutes after the sperm attachment the nucleolus of the egg disappears and at fifteen minutes the nucleus or germinal vesicle disappears. At about thirty to thirty-five minutes the first polar body is extruded and about ten minutes later the second is extruded (Plate I, Fig. *B*). In many cases the first polar body divides so that three appear. About one hour and ten minutes after sperm attachment the egg elongates and the furrow of the first cleavage appears and by one hour and twenty minutes the egg is in the two-cell stage. The second, third and fourth cleavages take place at half-hour intervals. This outline was made from the work of Tyler, confirmed by the writer's observations on several groups of eggs at 20° C.

The unfertilized egg appears to have a marked polarity as the bottom of the indenture seems to mark one pole (Plate I, Fig. *A*). Tyler considers this as the animal pole, for in 85 per cent of the eggs observed by him the polar bodies appear at a point less than 10° from the egg surface, which before fertilization was at the bottom of the indenture. Taylor (1931) however, states: "The immature egg apparently has no mark of polarity. Neither the eccentricity of the germinal vesicle nor the position of the one or more depressions could be correlated with the subsequent place of extrusion of the polar bodies, as will be stated in some detail in later paragraphs."

The position of the axis of the spindle of the first cleavage and therefore the plane of the cleavage is determined by the axis of the egg and some other point. Tyler has found that the plane of the first cleavage passes within ten degrees of the point of entrance of the sperm in 71 per cent of 178 eggs observed. The high correlation between point of sperm entrance and the position of the first cleavage furrow is confirmed by the work of Taylor.

Development to the Sixty-Four-Cell Stage

From the two-cell to the sixteen-cell stage.—The cleavage furrow first appears at the animal pole of the egg and spreads to the vegetal pole, cutting the egg into two equal-sized cells. These are at first almost spherical, being only slightly flattened on their adjacent sides. During the resting stage they further flatten and elongate and at this time the spindles of the second cleavage may be seen (Plate I, Fig. *C*). The axes of the spindles are parallel, and in stained preparations the chromosomes may be indistinctly seen and around the ends of the spindles red granules are visible.

The second cleavage furrow also starts at the animal pole of each cell and then divides it into two. The egg is then in the 4-cell stage, the cells being equal in size and flattened on their adjacent sides (Plate I, Fig. *D*). The long axes of the cells are parallel with the axis of the egg and a distinct, equal-sided segmentation cavity lies between them. In *Thalassema*, Torrey describes the two spindles of the second cleavage as being not quite parallel so that the *A* and *C* cells are in contact at the upper pole and the *B* and *D* cells are in contact at the lower pole, the furrow at the upper pole being a segment of the first cleavage furrow. This method of cleavage was not observed either in the living or preserved eggs of *Urechis*.

Preceding the third cleavage the four cells elongate vertically and become somewhat more rounded horizontally so that the area of contact between the cells decreases. As the spindles form with their axes coinciding with the long axes of the cells, the upper ends of the cells rotate to the right, clockwise; and the lower ends rotate to the left, counter-clockwise (Plate I, Fig. *E*). The resulting cleavage is thus spiral and dexiotropic. The upper moiety is the first quartet of micromeres and the cells are slightly smaller than the lower macromeres (Plate I, Fig. *F*). There is a rather brief resting period during which the cells become flattened on their adjacent sides and the whole egg becomes more spherical.

Both the macromeres and micromeres then elongate, and their upper ends rotate to the left, counter-clockwise (Plate II, Fig. *G*). The resulting cleavage is therefore spiral and leiotropic and is somewhat unequal. The first quartet now consists of eight cells, the lower cells, $1a^2$, etc., being somewhat smaller than the parent cells and the macromeres having divided to produce the second quartet, $2a$, etc., the cells of which are the same size as the daughter cells of the first

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PLATE I

All drawings were made with a camera lucida and have the same magnification. Figures *A* and *B* were drawn from living eggs. All other figures were drawn from fixed material and are therefore somewhat smaller.

FIG. *A*. The unfertilized egg showing the indenture or depression.

FIG. *B*. The fertilized egg with the polar bodies extruded, the fertilization membrane not being shown.

FIG. *C*. A semi-polar view of the 2-cell stage showing the parallel spindles of the second cleavage.

FIG. *D*. A polar view of the 4-cell stage showing the equal-sized cells and the equal-sided segmentation cavity.

FIG. *E*. A lateral view of the 4-cell stage as the cells divide to form the 8-cell stage.

FIG. *F*. A lateral view of the 8-cell stage.

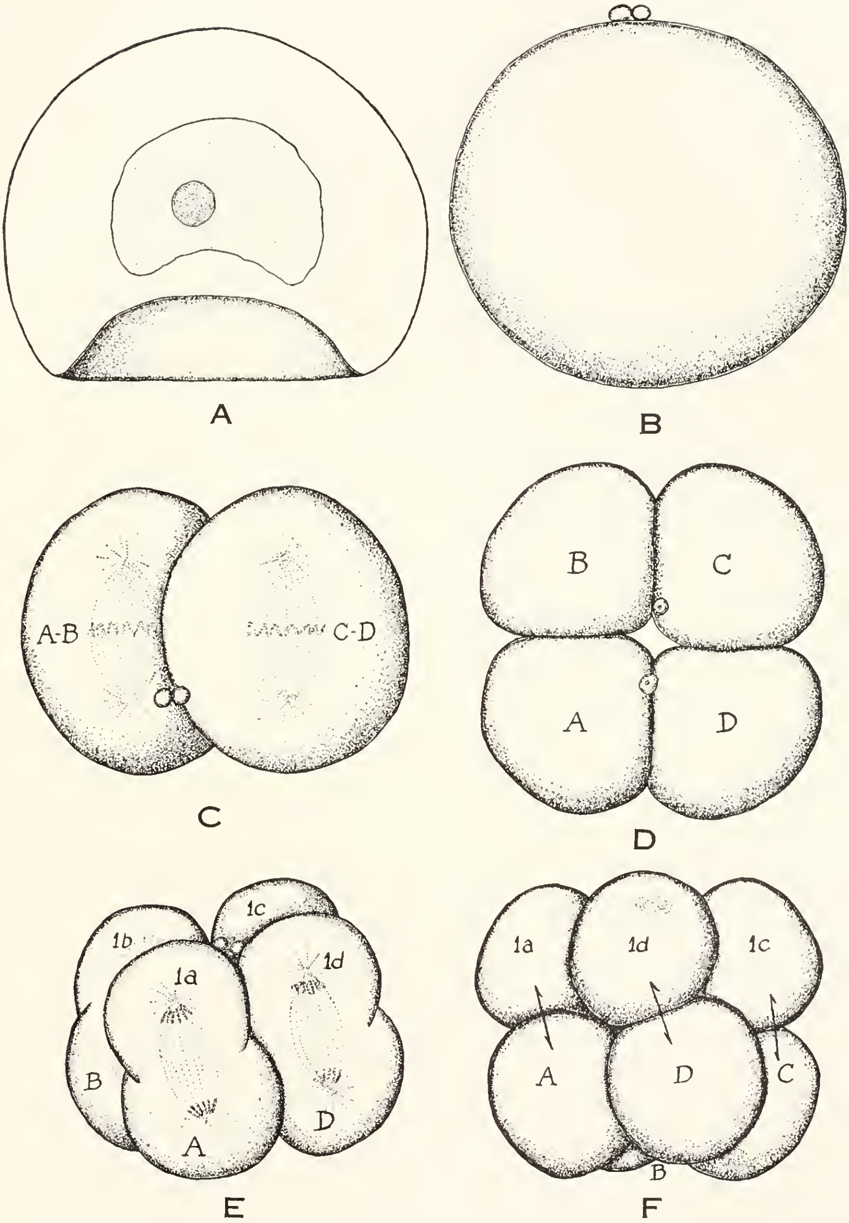
A
B
A-B
C-D
C
B
C
A
D
D
1c
1b
1a
1d
B
D
A
E
1a
1d
1c
A
D
C
B
F

Plate I

quartet (Plate II, Figs. *H*, *I*, and *J*). The daughter cells of the first quartet are the prototroch cells. The egg is radially symmetrical and during a rather protracted resting stage the cells become flattened on their adjacent sides. In the 8-cell stage the first quartet cells are to the right of the macromeres. In the 16-cell stage, however, because of the pressure of the newly-formed cells, the stem cells of the first quartet are pushed to the left of the macromeres.

The twenty-four-cell stage.—The next division is unequal and takes place simultaneously in the stem cells of the first quartet and in the macromeres (Plate II, Figs. *K* and *L*). It is dexiotropic and results in the formation of eight new cells, all markedly smaller than the parent cells and slightly smaller than the daughter cells of the preceding division. The egg is now in the 24-cell stage and consists of the first quartet of twelve cells, four newly formed ($1a^{1.2}$, $1b^{1.2}$, etc.), the second quartet of four cells, the newly-formed third quartet of four cells (*3a*, *3b*, etc.), and the four macromeres. During the rather brief resting stage the cells again flatten on their adjacent sides and by their pressure again push the stem cells of the first quartet to the right of the corresponding macromeres.

The thirty-two-cell stage.—This stage is produced by a division of the prototroch cells, $1a^2$, $1b^2$, etc., and the second quartet cells, *2a*, *2b*, etc. Both divisions are dexiotropic and about equal, but the division of the prototroch cells precedes that of the second quartet cells, the first being in a late stage of division when the latter is in an early stage (Plate III, Fig. *M*). In the resting period following this division the egg undergoes a change in shape. The cells of the first and second quartets become somewhat flattened and surround an enlarged segmentation cavity. The four macromeres remain in contact and have their adjacent sides almost vertical, the whole egg taking on a "mushroom shape" with the four macromeres appearing as four conspicuous cells at the base (Plate III, Figs. *N* and *O*).

This change in shape marks the second conspicuous deviation in the development of *Urechis* as compared with that of *Thalassema* as

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Plate II

Fig. *G*. A lateral view of the 8-cell stage as the cells divide to form the 16-cell stage.

Fig. *H*. A lateral view of the early 16-cell stage.

Fig. *I*. A polar view of the early 16-cell stage.

Fig. *J*. A lateral view of the 16-cell stage during the resting period. The extent of the segmentation cavity is indicated by the dotted line.

Fig. *K*. A lateral view of the 16-cell stage as the cells divide to form the 24-cell stage.

Fig. *L*. A lateral view of the 24-cell stage.

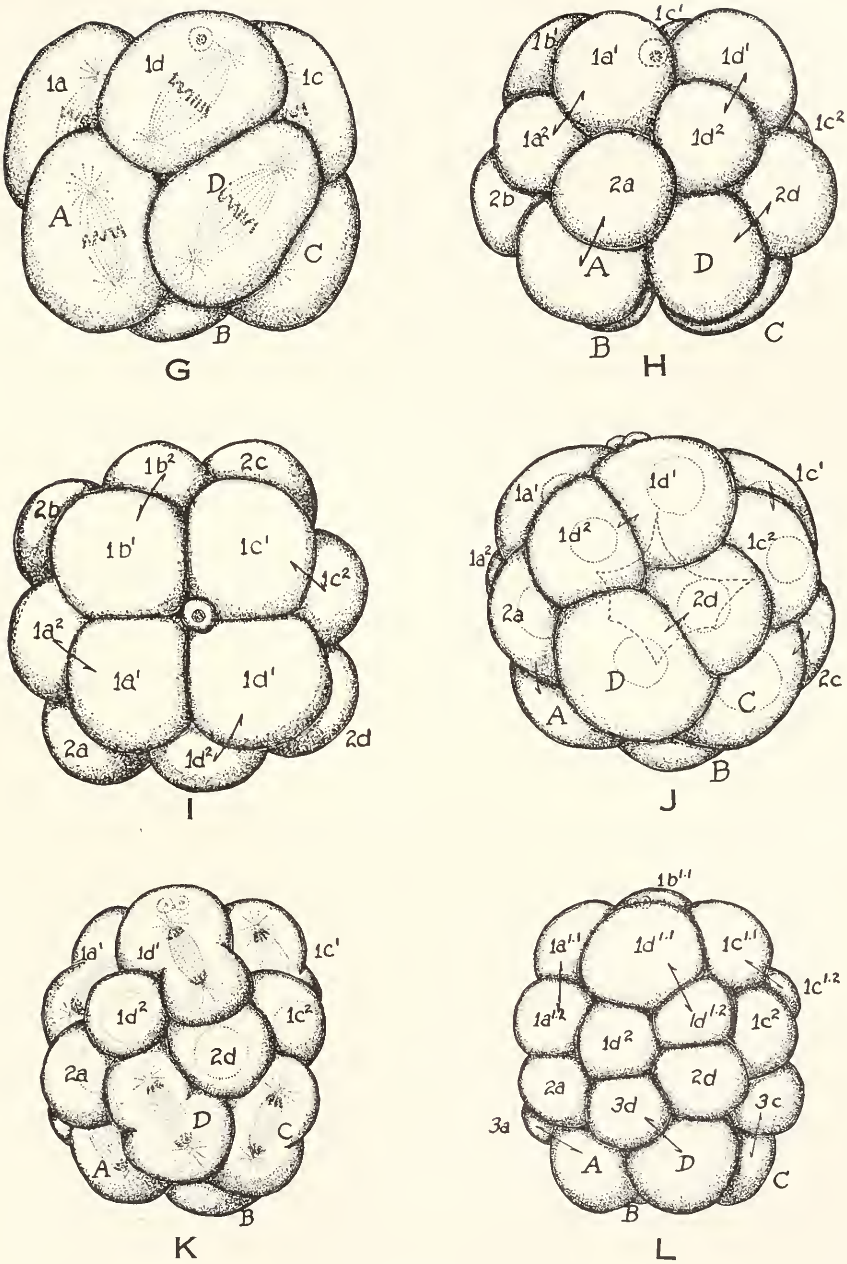

1a
1d
1c
A
D
C
B
G
1b'
1c'
1a'
1d'
1a²
1d²
1c²
2b
2a
2d
A
D
B
C
H
1b²
2c
2b
1b'
1c'
1c²
1a²
1a'
1d'
2a
1d²
2d
I
1d'
1a'
1c'
1d²
1a²
1c²
2a
2d
D
C
2c
A
B
J
1a'
1d'
1c'
1d²
1c²
2a
2d
D
C
A
B
K
1b¹·¹
1a¹·¹
1d¹·¹
1c¹·¹
1c¹·²
1a¹·²
1d¹·²
1d²
1c²
2a
2d
3a
3d
3c
A
D
C
B
L

Plate II

described by Torrey. The order of the cleavages has been the same and the general arrangement of the cells has been the same, but he figures the macromeres as being proportionally larger and he neither mentions nor figures the "mushroom shape" which characterizes the *Urechis* egg from this stage on to the rotating larval stage.

The thirty-six-cell stage.—This stage is formed by a leiotropic division of the macromeres to form the fourth quartet (Plate III, Figs. *N* and *O*). This division is unequal, the fourth quartet cells being even smaller than the cells of the second and third quartets. They are also pushed inward and flattened so that they form long cells extending radially into the egg and presenting a small surface to the outside.

This division is similar to that of *Thalassema* in that there is nothing in either the time of cleavage, the shape, size, or positions of the cells to distinguish the 4*d* or *M* cell from the other fourth quartet cells.

The forty-cell stage.—This stage is formed by a leiotropic division of the stem cells of the first quartet, $1a^{1.1}$, $1b^{1.1}$, etc. (Plate III, Figs. *P* and *Q*). The division is very unequal and the smaller polar cells, $1a^{1.1.1}$, $1b^{1.1.1}$, etc. form the apical rosette; and the larger daughter cells, $1a^{1.1.2}$, $1b^{1.1.2}$, etc. form the stem cells of the cross (Plate III, Figs. *P* and *Q*). The rosette cells lie in a depression between the cross cells. The cross cells also become rounded upward and there is a shifting of the $1a^{1.2}$, $1b^{1.2}$, etc. cells to a position between the outer ends of the cross cells. These cells, the $1a^{1.2}$, etc. may then be called the intergirdle cells. The terms "apical rosette," "cross cells," and "intergirdle cells" are used because they have the same origin and relative position as similar cells in *Thalassema*, their ultimate fate not being as yet known in *Urechis*.

The relatively smaller size and the position of the rosette cells in a depression is in contrast with the larger size and more superficial position of the cells in *Thalassema*. In this respect the apical rosette of *Urechis* resembles that of *Podarke*.

PLATE III

FIG. *M*. A lateral view of the 24-cell stage as the cells divide to form the 32-cell stage.

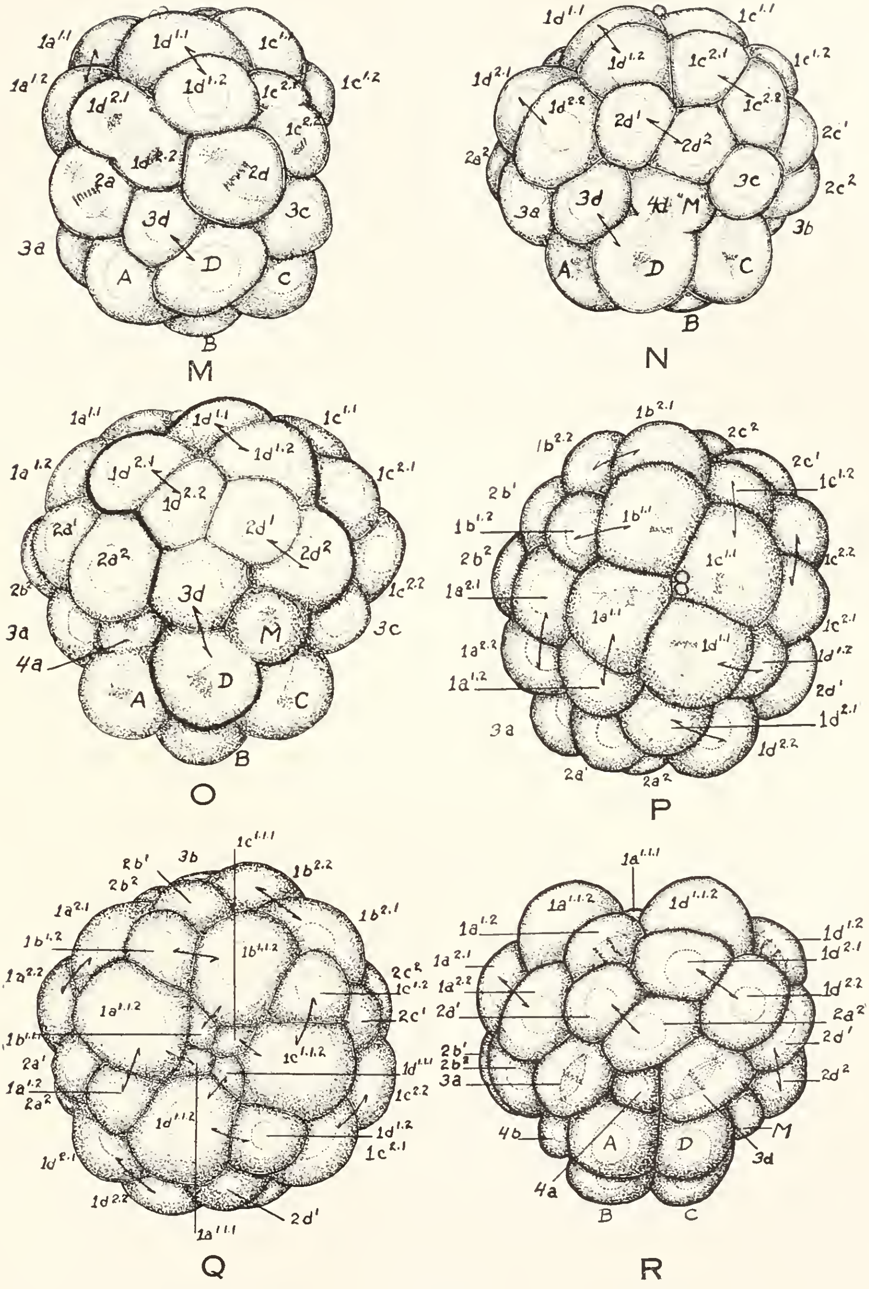
FIG. *N*. A lateral view of the 32-cell stage as the macromeres divide to form the 36-cell stage.

FIG. *O*. A lateral view of the 36-cell stage, showing the characteristic "mushroom" shape of the egg.

FIG. *P*. A polar view of the 36-cell stage showing the division of the micromeres to produce the 40-cell stage.

FIG. *Q*. A polar view of the 40-cell stage showing the apical rosette.

FIG. *R*. A lateral view of the 40-cell stage as the cells divide to form the 48-cell stage.


$1a^{1.1}$
$1d^{1.1}$
$1c^{1.1}$
$1a^{1.2}$
$1d^{1.2}$
$1c^{2.1}$
$1c^{1.2}$
$1d^{2.1}$
$1c^{2.2}$
$1d^{2.2}$
2a
2d
3d
3c
3a
A
D
C
B
M
$1d^{1.1}$
$1c^{1.1}$
$1d^{1.2}$
$1c^{2.1}$
$1c^{1.2}$
$1d^{2.1}$
$1d^{2.2}$
$1c^{2.2}$
$2d^{1}$
$2d^{2}$
$2c^{1}$
$2a^{2}$
3c
$2c^{2}$
3d
4d "M"
3a
3b
A
D
C
B
N
$1a^{1.1}$
$1d^{1.1}$
$1c^{1.1}$
$1a^{1.2}$
$1d^{1.2}$
$1c^{2.1}$
$1d^{2.1}$
$1d^{2.2}$
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$2d^{1}$
$2a^{2}$
$2d^{2}$
$2b^{1}$
$1c^{2.2}$
3d
3a
3c
4a
M
A
D
C
B
O
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$1b^{2.2}$
$2c^{2}$
$2c^{1}$
$1c^{1.2}$
$2b^{1}$
$1b^{1.2}$
$1b^{1.1}$
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$1c^{2.1}$
$1a^{2.2}$
$1d^{1.1}$
$1d^{1.2}$
$1a^{1.2}$
$2d^{1}$
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$1d^{2.2}$
$2a^{1}$
$2a^{2}$
P
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3b
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$1d^{2.2}$
$2a^{1}$
$2a^{2}$
$2d^{1}$
$2b^{1}$
$2b^{2}$
3a
$2d^{2}$
4b
M
A
D
3d
4a
B
C
R

Plate III

The forty-eight-cell stage.—This stage is formed by an almost simultaneous, leiotropic division of the third quartet cells and the intergirdle cells, the division of the intergirdle cells generally preceding that of the third quartet (Plate III, Fig. *R*). The division of the intergirdle cells is somewhat unequal, the upper cells, lying between the cross cells, being a little larger. The division of the third quartet is also unequal, the upper cells in this case being slightly smaller and rather deeply imbedded between the surrounding prototrochal and second quartet cells.

The fifty-six-cell stage.—This stage is formed by simultaneous, leiotropic, and equal divisions of the prototrochal cells, $1a^{2 \cdot 1}$, $1a^{2 \cdot 2}$, etc. (Plate IV, Fig. *S*). The order of these last two cleavages is different in *Thalassema* and *Urechis*. In *Thalassema* there is a division of the third quartet followed by an almost simultaneous division of the intergirdle and prototrochal cells. In *Urechis* the first division takes place simultaneously in the intergirdle and third quartet cells and the second is in the eight prototrochal cells only.

The sixty-four-cell stage.—This stage is formed by a division of the four cross cells, $1a^{1 \cdot 1 \cdot 2}$, etc., and the lower moiety of the second quartet cells, $2a^2$, etc. (Plate IV, Fig. *T*). These divisions are important as they mark the end of the purely radial cleavages and initiate the bilateral and morphogenic cleavages. The divisions follow exactly those in *Thalassema* and the symmetry is probably the same. In two adjacent cells of the cross, $1c^{1 \cdot 1 \cdot 2}$ and $1d^{1 \cdot 1 \cdot 2}$, a meridional, equal division occurs which is shortly followed by an unequal, leiotropic division of the other two cross cells, $1a^{1 \cdot 1 \cdot 2}$ and $1b^{1 \cdot 1 \cdot 2}$. This later division is almost meridional, the axes of the spindles being inclined only slightly to the left. The smaller cells are budded off toward the center and lie against the rosette cells (Plate IV, Fig. *U*). Bilaterality is now clearly established. However, the relation between the plane of the first cleavage and this plane of symmetry; and the relation between this plane of symmetry and that of the larva has not been determined at this time.

PLATE IV

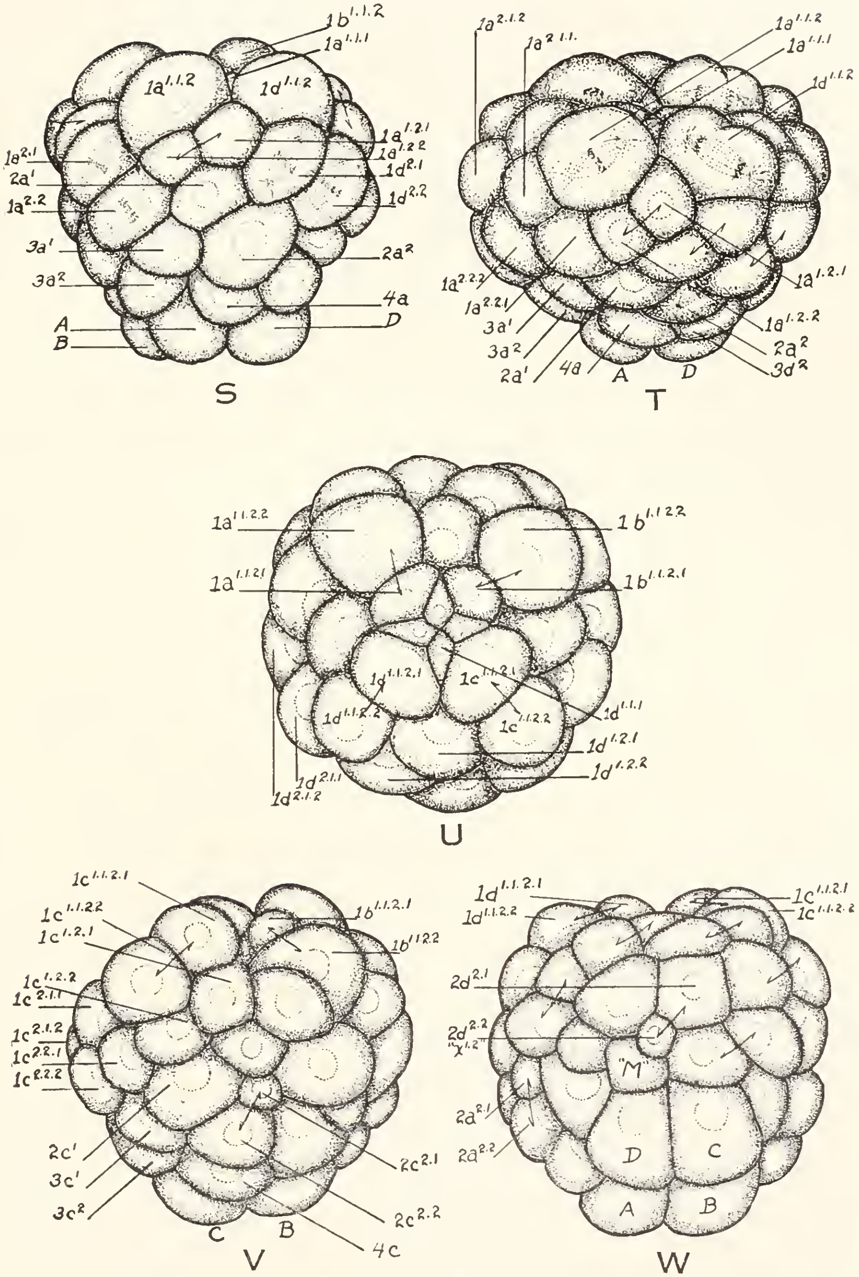
FIG. *S*. A lateral view of the 48-cell stage as the cells divide to form the 56-cell stage.

FIG. *T*. A lateral view of the 56-cell stage as the cells divide to form the 64-cell stage.

FIG. *U*. A polar view of the 64-cell stage showing the bilateral symmetry.

FIG. *V*. The right side of the 64-cell stage showing the $2c^{2 \cdot 2}$ cell as being larger than the $2c^{2 \cdot 1}$ cell. This is also the case in the division of the $2a^2$ and $2b^2$ cells.

FIG. *W*. A posterior view of the 64-cell stage showing the $2d^{2 \cdot 2}$ cell as being smaller than the $2d^{2 \cdot 1}$ cell. This marks the posterior side of the larva.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$1d^{2.2}$
$1a^{2.1}$
$2a^{1}$
$1a^{2.2}$
$3a^{1}$
$3a^{2}$
$2a^{2}$
$4a$
A
D
B
S
$1a^{2.1.2}$
$1a^{2.1.1.}$
$1a^{1.1.2}$
$1a^{1.1.1}$
$1d^{1.1.2}$
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$1a^{2.2.2}$
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$3a^{2}$
$2a^{1}$
$4a$
A
D
$1a^{1.2.2}$
$2a^{2}$
$3d^{2}$
T
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$1a^{1.1.2.1}$
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$1d^{1.1.1}$
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$1d^{2.1.1}$
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$1c^{1.1.2.2}$
$1c^{1.2.1}$
$1b^{1.1.2.1}$
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$1c^{1.2.2}$
$1c^{2.1.1}$
$1c^{2.1.2}$
$1c^{2.2.1}$
$1c^{2.2.2}$
$2c^{1}$
$3c^{1}$
$3c^{2}$
$2c^{2.1}$
$2c^{2.2}$
$4c$
C
B
V
$1d^{1.1.2.1}$
$1d^{1.1.2.2}$
$1c^{1.1.2.1}$
$1c^{1.1.2.2}$
$2d^{2.1}$
$2d^{2.2}$
"$X^{1.2}$"
"M"
$2a^{2.1}$
$2a^{2.2}$
D
C
A
B
W

PLATE IV

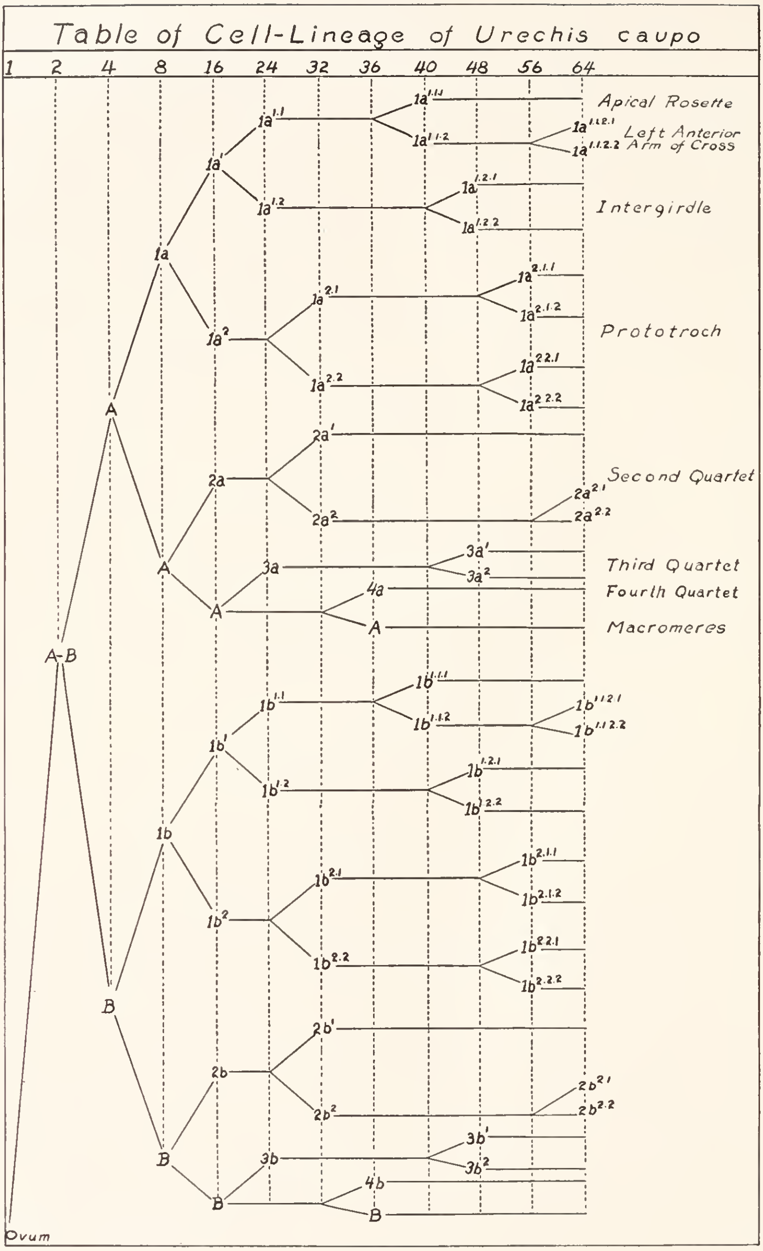
Table of Cell-Lineage of Urechis caupo
1
2
4
8
16
24
32
36
40
48
56
64
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Apical Rosette
$1a^{1.1}$
$1a^{1.1.2.1}$
Left Anterior Arm of Cross
$1a^{1.1.2}$
$1a^{1.1.2.2}$
$1a^{1}$
$1a^{1.2.1}$
$1a^{1.2}$
Intergirdle
$1a^{1.2.2}$
$1a$
$1a^{2.1.1}$
$1a^{2.1}$
$1a^{2.1.2}$
Prototroch
$1a^{2}$
$1a^{2.2.1}$
$1a^{2.2}$
$1a^{2.2.2}$
A
$2a^{1}$
Second Quartet
$2a$
$2a^{2.1}$
$2a^{2.2}$
$2a^{2}$
$3a^{1}$
A
$3a$
Third Quartet
$3a^{2}$
$4a$
Fourth Quartet
A
A
Macromeres
A-B
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$1b^{1.1.2.2}$
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B
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$2b^{2.1}$
$2b^{2}$
$2b^{2.2}$
$3b^{1}$
B
$3b$
$3b^{2}$
$4b$
B
B
Ovum

PLATE V

A table which shows the order of the first eleven divisions of the egg of *Urechis.* As the cleavages in all four quadrants are the same at this stage, only the anterior, *A* and *B*, quadrants are shown.

It has been assumed that the first, equal, meridional divisions of the cross cells take place in the posterior, *C* and *D*, quadrants as they do in *Thalassema*. This is further confirmed by the mode of division of the second quartet cells. In the $2a^2$, $2b^2$, and $2c^2$ cells the unequal, leiotropic divisions have the smaller cells budding off upwardly to become deeply imbedded between the surrounding cells. The $2d^2$ cell also divides unequally but with the smaller cell downward, overlying the $4d$ or *M* cell. This cell has been designated by Treadwell for *Podarke*, and Torrey for *Thalassema* as the $x^{1 \cdot 2}$ cell.

"Treadwell ('97 and '01) in his discussion of cell-homologies calls attention to the fact that this cell has the same origin in all the forms of annelids and molluscs that have been studied." (Quoted from Torrey.) The identity of the four quadrants and the plane of symmetry is thus established before the division of the *M* cell.

The sixteen prototrochal cells have a rather superficial position on the surface of the egg and do not extend inward to the segmentation cavity. The fertilization membrane persists around the entire egg but at the animal pole has a depression that brings it in contact with the apical rosette cells.

Development to the 64-cell stage takes five and one-half to six hours at 18° to 20° C. Cilia develop on the prototroch rather rapidly between six and a quarter and six and one-half hours and almost immediately begin to beat, causing the egg to rotate. The longer apical cilia develop later.

CONCLUSION

Except for the shape of the egg and differences in the relative sizes of some of the cells the egg of *Urechis* at the 64-cell stage is identical with that of *Thalassema* and similar to the eggs of other annelids with equal cleavages.

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POLYVITELLINE EGGS AND DOUBLE MONSTERS IN THE POND SNAIL LYMNÆA COLUMELLA SAY

CHARLES P. WINSOR AND AGNES A. WINSOR
Department of Biology, School of Hygiene and Public Health,
Johns Hopkins University

The occasional occurrence of gastropod eggs containing more than one embryo is more or less familiar to workers with these forms. Such embryos are normally separate; but occasionally conjoined twins are found, and more rarely triplets, and instances of as many as five conjoined embryos have been reported. The literature on this subject has been well reviewed by Pelseneer (1920) and Crabb (1931), so that no extended historical discussion seems necessary. Our purpose is merely to present certain material which seems to us useful in supplementing and perhaps in modifying the conclusions of other workers.

The material here presented consists of the total egg production throughout life of a population of (initially) 400 snails of the species *Lymnæa columella* Say. The wild parents of these snails were collected in two ponds in the vicinity of Baltimore, designated here as the Falls Road pond and the Boyce Avenue pond. In addition to these wild ancestors of known origin, two snails isolated from laboratory aquaria furnished eggs for this experiment; nothing is known concerning their origin. These animals were isolated in the laboratory in finger bowls with about 150 ml. of spring water, fed with leaf lettuce, and their eggs collected daily. The eggs so obtained were allowed to develop for about a week, at which time healthy-appearing clutches were selected for the experimental population. These eggs were removed from the capsule and placed in finger bowls with spring water. Each clutch of eggs provided twenty eggs, which were distributed among six finger bowls, one at density ten and five at density two per bowl. Leaf lettuce was used for food. Records of egg production were kept for each dish throughout the lives of the animals. Early in the experiment appreciable numbers of polyvitelline eggs appeared and were regularly entered in the records. Occasional double monsters were found; such eggs were saved and their development followed until the death of the embryos.

Table I summarizes the results obtained.

The facts regarding the production of polyvitelline eggs are summarized in Fig. 1. This chart shows the percentage of polyvitelline eggs laid in each finger bowl in the experiment. The parentage of the snails in each finger bowl is also shown, together with the geographical origin of the parents.

It is clear from an examination of the chart that there are very marked differences in the proportions of polyvitelline eggs occurring in different finger bowls; and that there is clear indication that the offspring of wild parents from the Falls Road pond, especially those from Nos. 2 and 4, laid a considerably higher proportion of polyvitelline eggs than those from the Boyce Avenue pond. There seems to be reason for supposing that these differences are the expression of some inherited character.

Table I

Production of Polyvitelline Eggs by Offspring of Different Wild Parents

Wild ancestor	No. of dishes	No. of snails	Total eggs	Total poly-vitelline eggs	Percentage poly-vitelline eggs	Eggs having given number of vitelli							
						2	3	4	5	6	7	8	9 and over
1	5	8	3,570	58	1.62	47	9	2	—	—	—	—	—
2	17	42	34,199	1,453	4.25	1,079	272	63	15	4	8	3	9
4	16	35	37,761	1,631	4.32	1,166	324	89	20	13	6	5	8
6	2	2	3,031	146	4.82	103	36	4	1	1	1	—	—
8	12	22	9,857	144	1.46	110	23	4	3	2	1	—	1
Totals 1–8 Falls Road	52	109	88,418	3,432	3.88	2,505	664	162	39	20	16	8	18
12	12	23	14,508	96	0.66	85	8	2	—	1	—	—	—
15	6	14	9,647	81	0.84	65	12	—	1	—	—	3	—
16	12	29	17,672	244	1.38	220	17	1	3	1	1	—	1
17	5	10	7,359	106	1.44	83	14	4	1	3	—	—	1
Totals 12–17 Boyce Ave.	35	76	49,186	527	1.07	453	51	7	5	5	1	3	2
A2	7	10	13,411	227	1.69	209	8	3	3	1	—	—	3
A3	6	11	13,369	73	0.55	63	3	7	—	—	—	—	—

Notes: Column 1 shows the wild ancestor. Numbers 1 to 8 inclusive were taken from the Falls Road pond; numbers 12 to 17 from the Boyce Avenue pond. Numbers A2 and A3 were isolated from a laboratory aquarium culture; their wild origin is unknown.

This conclusion differs from that of Crabb, who holds that there is no indication that the production of polyvitelline eggs is influenced by hereditary factors. The data given by the Crabbs (1927) seem to us, however, consistent with the notion that inheritance does play a part in the phenomenon. Neither their data nor ours, however, are adequate to suggest any Mendelian mechanism.

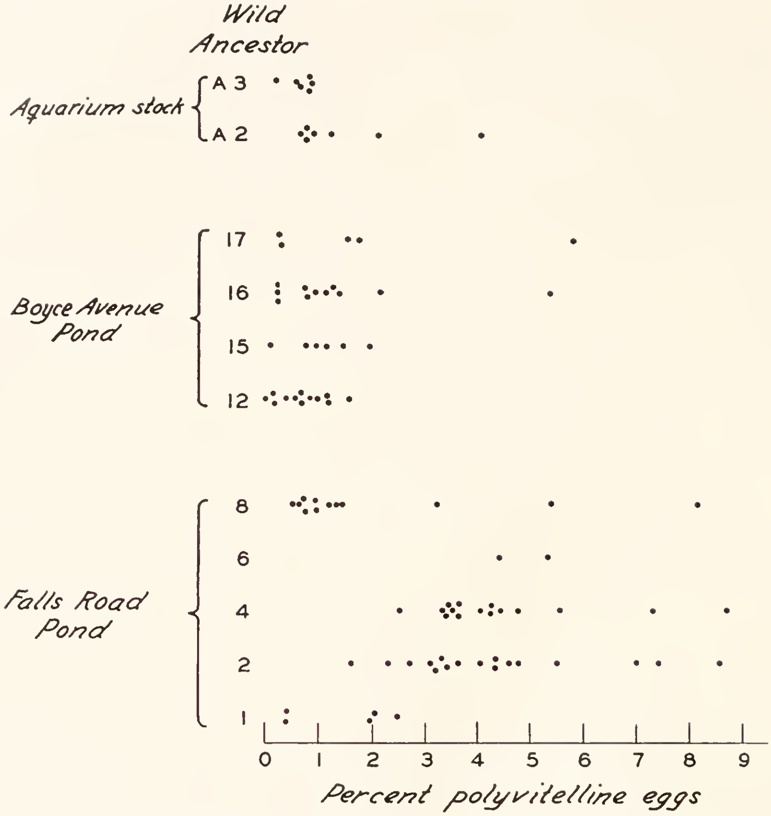


FIG. 1. Chart showing proportion of polyvitelline eggs in different finger bowls, segregated by ancestry.

A paper by Tur (1910) (not cited by Crabb) is of interest in this connection. He found, in *Philine aperta*, that there were distinct local races, some of which produced polyvitelline eggs with some frequency and regularity, while animals from other localities rarely or never laid such eggs.

EXPLANATION OF PLATE

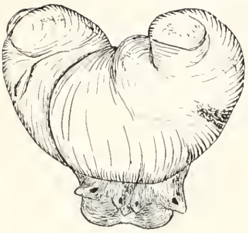
FIGS. 1–3. Lateral fusion with separate shells. Three views. Parent No. 27, grandparent No. 2.

FIGS. 4–5. Dorsal fusion with separate shells. Two views. Parent No. 7, grandparent No. 4.

FIG. 6. Visceral masses fused, single shell. Parent No. 12, grandparent No. 4.

FIGS. 7–8. Triple monster, shell fused. Parent No. 26, grandparent No. 2.

FIG. 9. Dorsal fusion, single shell. Parent No. 11, grandparent No. 4.



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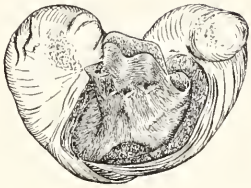

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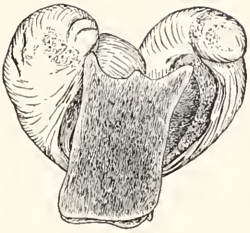

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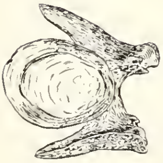

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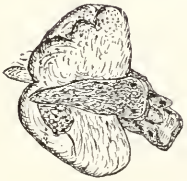

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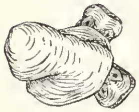

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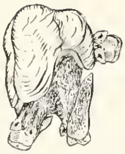

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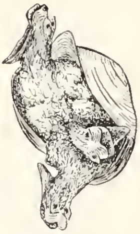

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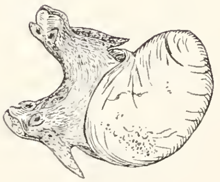

9

DOUBLE MONSTERS

Double monsters in considerable numbers were observed in our material. Drawings of typical specimens (made for us by the laboratory artist, Mr. Arthur Johansen) are shown in Plate I. As regards the anatomical details of these monsters, we can add nothing of importance to Pelseneer's account. So far as our observations went, they agreed with his.

It will be noted that the wild ancestors giving rise to these double monsters came almost entirely from the Falls Road pond, as would be expected in view of their apparently certain origin through fusion of embryos. Actually, of 32 double and triple monsters, 28 (of which one was triple) had a Falls Road origin; one was derived from Boyce Avenue stock; and three (one triple) from laboratory stock of unknown origin.

SUMMARY

Data are presented regarding the production of polyvitelline eggs and double monsters through the entire life of a group of *Lymnæa columella*. It is held that the data show that hereditary factors are concerned.

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PELSENEER, P., 1920. Les variations et leur hérédité chez les mollusques. *Mém. Ac. Roy. Belg., Cl. Sci.*, Coll. in—8°, **5**: 1 (pp. 308–339 deal with double monsters).

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Detailed bibliographies will be found in Crabb (1931) and Pelseneer (1920).

STUDIES ON AMPHIBIAN METAMORPHOSIS

X. HYDROGEN-ION CONCENTRATION OF THE BLOOD OF ANURAN LARVÆ DURING INVOLUTION[1]

O. M. HELFF

Department of Biology, University College, New York University

Introduction

The metamorphosis of anuran larvæ, aside from the striking embryological and growth phenomena exhibited, presents perhaps an even more interesting series of degenerative changes, involving cellular autolysis and various histolytic effects, which in some cases results in the total atrophy of an organ. The range of tissues so affected and the degree to which they degenerate is subject to considerable variation, however. Thus we find the tail, the gills, and the areas of opercular integument through which the fore-limbs emerge undergoing complete atrophy resulting in the total resorption of the tissues concerned, with the exception of the gills which are represented in the adult by small spherical bodies, the "Kiemenreste," whose function, if any, is unknown. On the other hand, organs such as the intestines, stomach, and pancreas which undergo a reorganization in the development of the adult condition, are subject to a lesser degree of atrophy, although the amount of cellular histolysis is very great. Contrasted to the above two categories of degenerating tissues, we find those in which relatively little degeneration occurs as in the tissues of the jaws during the shedding of larval teeth, the early degenerative changes of integument in the formation of the tympanic membranes, and the general atrophy of the musculature of the back, the latter being subject to approximately a 38 per cent reduction in volume during metamorphosis. Although relatively little, if any, information is available regarding the histological changes operating in the case of the jaw tissues, pancreas, and gills, more definite data can be found in relation to the other organs and tissues as cited above. For such information the reader is referred to the papers of Barfurth (1887), Bataillon (1891), Bradley (1922), Clausen (1930), Helff (1926) (1928) (1931*a*), Helff and Clausen (1929), Kuntz (1922) (1924), Lindeman (1929*a*), Mercier (1906), Morse (1918), and Van Der Jagt (1929).

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[1] The expenses entailed in this investigation have been partially met by a grant of money from the Bache Fund of the National Academy of Sciences.

Although the endocrine glands, notably the thyroid and anterior pituitary, are generally conceded to furnish the initial stimuli for amphibian metamorphosis, relatively little information is available in respect to their mode of action. It seems quite certain, however, that certain developments and degenerative processes are not brought about by the endocrine hormones acting directly on the tissue concerned through the medium of the blood stream. Thus the dedifferentiation of integument to form the tympanic membranes is due to contact of the integument with the developing annular tympanic cartilage and columella (Helff, 1928 and 1931), while the total histolysis of opercular integument as it occurs in the formation of the fore-limb perforations is the result of integumentary contact with the atrophying tissue of the gills (Helff, 1926). On the other hand, the development and degeneration of tissues following transplantation to foreign regions strongly suggests the operation of metamorphic influences brought into play through the blood supply to the transplant. The development of the tongue (Helff, 1929), the columella (Helff, 1931*a*), the dermal plicæ (Helff, 1931*b*), the fore-limbs (Helff, 1926), the hind-limbs (Schubert, 1926), the adult pigment pattern (Lindeman, 1929*a*) and the nictitating membrane (Lindeman, 1929*b*) are all cases in point in which the developmental stimulus is through the blood stream. The degenerative changes which have been shown to occur normally upon transplantation to foreign regions include the histolysis of tail integument (Lindeman, 1929*a*, and Clausen, 1930), and the histolysis of tail muscle (Helff and Clausen, 1929). Indications also point to the probability that future work will add the atrophy of the gills, intestines, and pancreas to this latter group.

While it is possible that all of the developmental and atrophic changes which occur during amphibian metamorphosis may be due to hormonic influences in the blood stream, it seems rather improbable that such will ultimately prove to be the case in relation to the partial or total histolysis of larval organs. Due to the considerable mass of work that has been done on various tissues in widely divergent animal groups in relation to the biochemical factors associated with and favorable to the autolysis of living tissues "in vivo" and "in vitro," one cannot escape the implication that the histolysis of larval organs and tissues in the amphibian is due to a more immediate and plausible causative factor which is the result, primarily, of course, of the metamorphic hormones. An obvious factor and one that has been shown to be closely correlated with the autolysis and atrophy of a wide variety of tissues in other classes of animals, is that of the hydrogen-ion concentration of the medium in which the autolysis occurs. Briefly, it may be stated that in general the increasing of the hydrogen-ion concentration of the medium above a certain level either tends to activate the proteolytic enzymes concerned in autolysis or else brings about changes in the tissue proteins themselves which render them digestible by the omnipresent enzymes.

The possibility of a lowering in the pH of the blood during amphibian metamorphosis has been suggested by many writers as a likely explanation for many of the histolytic processes which occur. Thus Barfurth (1887), Bataillon (1891), Mercier (1906), Morse (1918), and Bradley (1922) all suggest the probability that the tissues of the larval tail undergo autolysis in response to a localized increase in hydrogen-ion concentration, the result of a partial or total occlusion of the dorsal aorta resulting from pressure exerted by the rapidly growing urostyle. Although their assumption of a lowered pH as the immediate causative factor may have been correct, the importance of the developing urostyle in this connection was found to be negligible as shown by subsequent work. In this connection it may be stated that the writer (1930) was able to show that the process of tail resorption was not inhibited in any way in the complete absence of the urostyle, the latter having been extirpated while still in the anlage stage prior to the onset of larval involution. It was also shown by Lindeman (1929*a*) that tail integument previously transplanted to the back underwent normal histolysis during metamorphosis at the same time the integument on the tail autolyzed. The same result was obtained by Helff and Clausen (1929) working with tail muscle.

A consideration of the foregoing brief survey regarding the problem of autolysis during larval involution clearly suggests that the question of hydrogen-ion concentration of the blood before, during, and after metamorphosis becomes a problem of considerable interest and importance. As far as the writer is aware, there is no previously published work relating to the hydrogen-ion concentration of larval blood either before or during metamorphosis. Numerous tests have been made on the blood of the adult amphibian, however, in relation to various environmental and experimental factors. In this relation the reader is referred to the papers of Rohde (1920), Hertwig (1927), Kamm (1930), et al. The present paper, therefore, relates the results of hydrogen-ion tests as made on the blood of non-metamorphosing larvæ, larvæ in various stages of involution, and young frogs shortly after the completion of metamorphosis.

Materials and Methods

The initial stock used for all hydrogen-ion tests were large second-year *Rana clamitans* larvæ obtained at regular intervals from a common source near Cincinnati, Ohio, during the winter and spring months of 1930 and 1931. The selection of *Rana clamitans* larvæ was based on two points both favorable to the work at hand; first, in that the large size of the tadpoles insured a sufficient quantity of blood for testing purposes and second, that due to the neotenous nature of the species and the fact that they were second-year larvæ, normal metamorphosis could be induced when placed in water at room temperatures during the spring months especially. Shipments of larvæ, when received, were first placed in a large tank in order to acclimate them to the new water medium and also to allow the less hardy to die out. Under laboratory conditions larvæ also frequently tend to develop edema and red-leg, cases of which were always eliminated during this preliminary quarantine period. In testing the normal or Stage 1 larvæ, the tadpoles were first isolated in individual bowls of water for a period of at least three days prior to the hydrogen-ion testing. In obtaining the various states of metamorphosis tested, Stage 1 larvæ were likewise isolated in individual bowls of water and allowed to metamorphose normally. In both cases no food was given to the animals. The time required at room temperatures for complete metamorphosis to occur following the onset of involution varied from four to seven weeks.

The method employed in obtaining samples of blood for testing purposes was as follows: The larvæ or newly metamorphosed frogs were first fastened, dorsal side down, to a moistened pad of cloth by means of rubber bands. It may be said in this connection that the elimination of the use of an anæsthetic in the securing of the blood samples was thought to be advisable in that the pH of the blood might be altered by such a procedure. Two incisions were now made through the integument of the anterior ventral surface; one running from side to side just anterior to the level of the heart, and the other along the mid-ventral line from the middle of the first incision anterior to the rim of the lower jaw. The two flaps of integument were now pulled aside, laterally, so as to expose the general region anterior to the heart. In the case of larvæ this exposes the pericardial sac, while in the young frog it is first necessary to resect certain underlying muscular layers before the pericardium is brought to view. Following the exposure of the pericardial sac, the latter is cut at a point to expose the truncus arteriosus. In so doing, care must be exercised not to cut too far posterior or else premature bleeding may occur due to injury of the anterior abdominal vein. The cutting of the pericardial sac liberates, especially in normal larvæ, a considerable amount of lymph which quickly flows out, however. The lymph is now carefully removed from the cavity so prepared immediately anterior to the heart.

The truncus arteriosus is now cut through using a small Noyes' iris scissors and blood quickly flows out filling the prepared cavity anterior. The blood is quickly drawn up into a pipette which has been previously rinsed with a 2 per cent solution of sodium oxalate. The small amount of oxalate solution adhering to the walls of the pipette is sufficient to prevent the clotting of the blood. The blood is now immediately transferred to a small vial and the hydrogen-ion concentration test quickly made.

The determination of hydrogen-ion concentration was made by means of the quinhydrone method. In this regard it seems advisable to mention some of the more important technical points found advantageous in securing greater accuracy with this method. Care must be exercised in the cleaning of the blood vials. The routine followed was to first thoroughly wash the vials and then rinse several times with hydrochloric acid followed by alcohol and distilled water. The vials were then allowed to dry thoroughly before using. Due to the small quantity of blood tested, very little quinhydrone had to be added. Solution of the latter in the blood was facilitated by gently tapping the vial for a few seconds. The platinum leaf electrode was next inserted to the bottom of the vial. By bending the platinum leaf at right angles to the supporting capillary glass tube, the former could be made to lie flat on the bottom of the vial and thus insure contact with the blood on both surfaces. Care should also be taken to carefully cleanse the test electrode between any two consecutive tests by rinsing thoroughly in hydrochloric acid, alcohol, and distilled water. The electrode should be dried carefully before using again. The agar bridges employed were of the usual type except that quite small tubing was necessary due to the small diameter of the blood vials. Care should be taken to cleanse the end of the agar bridge which has been immersed in the blood of the test vial, between individual tests. The quicker the e.m.f. is determined the better, while use of a room with little variation in temperature also makes for greater accuracy, especially where comparative results are desired. Finally, it is a good practice to standardize the set at frequent intervals by checking against a standard solution of 1/20 molar potassium acid phthalate which should give a pH of 3.98 at 25° C. In the present work such a standardization test was made between every two consecutive blood tests.

RESULTS

Blood pH of Normal, Non-metamorphosing Larvæ

The larvæ used for the Stage 1 tests were normal tadpoles in all respects which would not have begun metamorphosis for at least a

TABLE I

Measurements of pH of Blood of Rana clamitans at Different Stages in Metamorphosis

Stage 1 Normal larvæ			Stage 2 First metamorphic change			Stage 3 One fore-leg emerged			Stage 4 Complete metamorphosis		Stage 5 3 to 4 weeks after Stage 4	
Weight	Length*	pH	Weight	Length*	pH	Weight	Length*	pH	Weight	pH	Weight	pH
gms.	*mm.*		*gms.*	*mm.*		*ms.*	*mm.*		*gms.*		*gms.*	
8.0	15 × 105	7.54	5.3	18 × 95	7.41	2.7	23 × 83	7.18	2.4	7.29	1.5	7.24
7.7	8 × 103	7.54	6.4	20 × 97	7.36	4.7	32 × 92	7.19	2.7	7.18	2.2	7.19
7.4	14 × 100	7.43	5.6	17 × 88	7.38	3.6	31 × 85	7.35	2.0	7.16	1.7	7.14
8.1	13 × 105	7.52	5.8	19 × 95	7.45	4.3	37 × 95	7.28	2.5	7.19	1.9	7.15
7.0	15 × 93	7.49	4.9	21 × 95	7.47	3.3	28 × 85	7.20	1.8	7.23	1.7	7.18
8.2	10 × 105	7.54	5.2	22 × 97	7.40	4.0	27 × 90	7.28	2.1	7.10	2.4	7.22
8.6	12 × 105	7.54	4.7	19 × 98	7.44	4.0	33 × 88	7.32	2.3	7.24	2.3	7.18
7.4	8 × 102	7.54	4.8	19 × 100	7.34	3.4	30 × 82	7.23	2.5	7.17	1.8	7.19
7.6	9 × 102	7.52	4.6	16 × 90	7.39	5.0	33 × 94	7.28	2.5	7.13	2.4	7.11
7.4	10 × 92	7.45	5.2	18 × 96	7.42	5.1	36 × 97	7.34	3.0	7.26	2.1	7.26
7.2	13 × 98	7.47	5.0	19 × 97	7.40	4.7	32 × 85	7.31	2.7	7.19	1.9	7.19
8.2	17 × 100	7.49	4.7	20 × 99	7.37	3.8	24 × 85	7.24	2.3	7.20	2.0	7.14
7.2	14 × 101	7.53	4.9	19 × 96	7.44	3.9	26 × 91	7.21	2.4	7.16	2.1	7.16
6.7	8 × 98	7.43	5.1	21 × 97	7.37	4.1	25 × 87	7.26	2.0	7.18	2.0	7.20
6.7	8 × 102	7.50	4.8	21 × 99	7.33	4.5	34 × 92	7.28	2.6	7.25	2.5	7.24
7.2	11 × 98	7.54	5.4	18 × 97	7.41	3.7	30 × 90	7.33	2.2	7.22	2.1	7.25
7.6	10 × 101	7.49	4.6	22 × 100	7.28	3.9	28 × 88	7.25	1.9	7.17	2.0	7.28
8.1	9 × 102	7.52	5.0	21 × 94	7.35	4.4	31 × 91	7.31	2.5	7.26	1.8	7.15
7.4	13 × 94	7.47	5.1	17 × 95	7.44	3.6	30 × 93	7.27	2.3	7.24	1.4	7.23
7.7	12 × 100	7.52	4.7	19 × 96	7.38	4.5	28 × 91	7.24	2.4	7.18	2.1	7.18
7.3	15 × 102	7.53	4.9	19 × 99	7.36	3.8	25 × 86	7.28	1.9	7.23	1.7	7.12
7.6	10 × 101	7.48	5.5	18 × 93	7.45	3.7	32 × 96	7.30	2.7	7.27	1.9	7.15
8.1	12 × 104	7.50	5.2	20 × 94	7.44	3.9	23 × 84	7.24	2.3	7.17	2.0	7.11
7.3	9 × 99	7.53	4.4	21 × 97	7.31	4.1	27 × 86	7.33	2.6	7.20	1.8	7.12
7.8	11 × 103	7.49	4.8	19 × 95	7.33	4.3	28 × 92	7.23	2.1	7.18	1.5	7.14

* Hind-limb length × total length.

month. Their hind-limb lengths varied from 8 to 17 mm., while in total length the variation was between 92 and 105 mm. The weight of such individuals varied from 6.7 to 8.6 grams.

Of the various stages tested, Stage 1 larvæ were most favorable in respect to the quantity of blood obtainable. Although some variation was experienced in this regard, a sufficient amount was always present. On the average about one-tenth of a cubic centimeter could be secured per animal. Columns 1, 2, and 3 of Table I present the essential data for this group of tests. It will be seen that the range of pH was not great, being from a minimum of 7.43 to a maximum of 7.54 and representing a variation, therefore, of but 0.11 of a pH unit. The average pH of the twenty-five larvæ tested proved to be 7.50 ± .00459, while the standard deviation amounted to .034 ± .0032 (see Table II). We can thus consider a pH of 7.50 as typical for the blood of normal non-metamorphosing larvæ of the particular species being tested.

TABLE II

Means, Standard Deviations, and Probable Errors

Stage	Mean	Standard deviation
1	7.50 ± .00459	.034 ± .0032
2	7.39 ± .00650	.048 ± .0046
3	7.27 ± .00634	.047 ± .0045
4	7.20 ± .00607	.045 ± .0043
5	7.18 ± .00648	.048 ± .0046

Blood pH During Metamorphosis

The various stages of metamorphosis tested were obtained by allowing natural metamorphosis to take place. The isolation of Stage 1 larvæ in individual bowls of water at room temperature acted as a stimulus to transformation with the result that the onset of metamorphosis usually began in about three weeks following their transfer from the stock tanks of cold running water. All cases of edema and red-leg which occurred during metamorphosis were discarded. During the early stages of involution the mortality was relatively low. The acquiring of completely metamorphosed larvæ, however, involved a mortality of over 80 per cent, necessitating the isolation of over one hundred and twenty-five Stage 1 larvæ in the ultimate securing of twenty-five Stage 4 animals. To obtain twenty-five Stage 5 animals, it was necessary to isolate a still larger number of Stage 1 larvæ, since the mortality was even greater in that about one-fourth of the Stage 4 animals died within three to four weeks following the attainment of that stage. In all, some four hundred and thirty Stage 1 larvæ were isolated in securing twenty-five cases each of Stages 1, 2, 3, 4, and 5.

Stage 2.—Stage 2 larvæ were tadpoles which had attained early metamorphic changes characterized by a definite acceleration in fore- and hind-limb growth, the loss of the full-bellied appearance typical of Stage 1 larvæ, a slight anterior-posterior atrophy of the tail with pronounced histolysis of the dorsal and ventral finny portions, and a general darkening of integumentary pigmentation. The hind-limbs varied between 17 and 22 mm. in length, and total length between 88 and 100 mm. The weight varied between 4.6 and 6.4 grams.

The fourth, fifth, and sixth columns of Table I present the essential data for Stage 2 tests. It will be noted that the range in pH was somewhat greater than in the case of the Stage 1 tests, being from 7.28 to 7.47 or a variation of 0.19 of a pH unit. The average pH of the twenty-five larvæ tested amounted to 7.39 ± .00650, the standard deviation being .048 ± .0046. The results are clear, therefore, in that a definite change in pH from 7.50 to 7.39, representing a drop of 0.11 of a pH unit, occurs during this early stage of metamorphosis.

Stage 3.—Stage 3 larvæ represented a more pronounced stage of larval involution. At this stage the left fore-limb had just emerged, while the hind-limbs were considerably increased in length. The tail had undergone still further atrophy and the intestines were rapidly being transformed into the adult type. The hind-limb lengths varied between 23 and 37 mm., and total length between 82 and 97 mm. The drop in weight was especially pronounced, the larvæ varying between 2.7 and 5.1 grams.

The ninth column of Table I presents the hydrogen-ion concentrations determined for Stage 3 larvæ. The range in pH is from 7.18 to 7.35 or a variation of 0.17 of a pH unit. The average pH of the twenty-five tests amounts to 7.27 ± .00634 with a standard deviation of .047 ± .0045. A still further drop of 0.12 of a pH unit on the average has apparently occurred, therefore, due to the more advanced stage of metamorphosis attained.

Stage 4.—Stage 4 individuals were characterized by practically complete metamorphosis. The animals were fully metamorphosed except for the persistence of a small 5–10 mm. tail stump and the lack of tympanic membrane development. Pigmentation, jaw development, and sitting posture were typically frog-like. Body and hind-limb measurements were not made, but the weight was found to vary between 1.8 and 3.0 grams.

The individual pH determinations of Stage 4 animals are listed in column 11 of Table I. The range is from 7.10 to 7.29, or a variation of 0.19 of a pH unit. The average pH amounted to 7.20 ± .00607 and the standard deviation was .045 ± .0043 (Table II). The average pH therefore indicates a still further drop in concentration amounting, on the average, to 0.07 of a pH unit.

Blood pH Following Metamorphosis

Newly-metamorphosed young frogs were selected for the final group to be tested in order to determine whether or not the drop in pH, as it occurs during metamorphosis, is a transient phenomenon or is an actual adjustment of the blood to a hydrogen-ion concentration typical of the adult of the species concerned. The weights of Stage 5 animals varied between 1.4 and 2.5 grams. The average weight was somewhat less than that of Stage 4 animals, due no doubt to the fact that they had been without food for from 3 to 4 weeks.

Column 13 of Table I lists the individual pH determinations made. The range is from 7.11 to 7.28 or a variation of 0.17 of a pH unit. The average pH of the entire group amounts to 7.18 ± .00648 with a standard deviation of .048 ± .0046 (see Table II). The difference between pH 7.20 (the average for Stage 4) and 7.18 (the average for Stage 5) is insignificant and well within the probable and experimental errors. Hence it seems probable that this pH represents, approximately, the hydrogen-ion concentration of the adult frog's blood for the species in question. This point, however, is a debatable one and will be further taken up in the next section of this paper.

DISCUSSION

The variation in blood pH between Stage 1 larvæ, although not excessive, calls for some explanation. The greatest difference between any two of the twenty-five larvæ tested amounted to 0.11 of a pH unit. This variation can be partially accounted for by the limitations of the quinhydrone method which admits of an accuracy to within only 0.04 of a pH unit. It seems quite probable, however, that a definite amount of variation actually exists between various individuals of the same developmental stage in that even greater differences have been recorded in the adult frog. Thus Kamm (1930), working with fully-grown *Rana esculenta* and *Rana temporaria*, records blood readings ranging from pH 7.36 to pH 7.61 in cases of starved animals. On the same species Hertwig (1927) obtained variations ranging from pH 7.36 to pH 7.59, while Rohde (1920) recorded a minimum pH of 6.32 and a maximum pH of 7.13. Kamm has pointed out, however, that the extreme variations as recorded by Rohde were probably due to certain technical errors. The wide variations as recorded by Kamm and

Hertwig, however, give evidence of the differences in hydrogen-ion concentration of the blood that exist between individual adult frogs of the same size and developmental condition. Apparently the blood of the amphibian is very poorly buffered as compared with the condition found in the mammals. This point is strongly emphasized by Kamm's (1930) work in which the pH of frog's blood was quickly lowered by feeding with boric acid and as quickly raised by sodium carbonate feeding. It seems quite likely, therefore, that the blood pH differences as recorded in the present paper for normal larvæ represent actual differences between individuals, the result of a relatively poor controlling mechanism for maintaining a constant hydrogen-ion concentration of the blood stream. In this connection it should be stated, however, that the comparatively greater extremes recorded between individuals in Stages 2, 3, 4, and 5 were no doubt partially due to the difficulty of selecting twenty-five animals of exactly the same developmental stage.

Table III

Comparison of Mean Differences and their Probable Errors

Stages compared	Differences between means	Probable error of the difference	Difference / Probable error of the difference	Average percentage decrease between means
				per cent
1 and 2........	.11	.007957	13.8	1.5
2 and 3........	.12	.009080	13.2	1.6
3 and 4........	.07	.008777	8.0	1.0
4 and 5........	.02	.008879	2.3	.3

The results of the present paper clearly indicate that an actual lowering of blood pH occurs during metamorphosis. The drop in pH would appear to be a gradual one, although somewhat more pronounced in the earlier stages of involution. That the various differences recorded between the means of Stages 1, 2, 3, and 4 are significant ones is shown by an analysis of such differences in relation to their respective probable errors (see Table III). Thus the difference between the means of Stages 1 and 2 or 0.11 of a pH unit is 13.8 times its probable error while the differences between Stages 2 and 3, and 3 and 4, or 0.12 and 0.07 of a pH unit, respectively, are 13.2 and 8.0 times their probable errors. Hence the differences as recorded are significant and represent an actual increase in hydrogen-ion concentration.

The difference between the means of Stages 4 and 5 (see Table III), or 0.02 of a pH unit, being only 2.3 times its probable error indicates that no significant change in hydrogen-ion concentration of the blood had occurred within 3 to 4 weeks following the close of the metamorphic period. Whether this represents the condition typical of young frogs newly metamorphosed in a natural environment is a questionable point. The experimental animals in the laboratory were not fed and hence were in a starved condition when tested. Young frogs in their natural outdoor environment would be feeding at this stage and increasing their weight instead of losing weight as was typical of the experimental animals (see Stage 4, Table I). In this relation it may be stated that feeding in the fully-grown frog apparently affects blood pH; according to Kamm (1930) who found the pH higher in the case of laboratory-starved animals as compared with freshly caught, well-nourished frogs. It seems quite probable, however, that the hydrogen-ion concentration of the blood may decrease somewhat in *Rana clamitans* as the latter attain their full size during successive years of growth, especially since Kamm (1930) and Hertwig (1927), have recorded concentrations well above pH 7.3 for fully-grown adults of *Rana esculenta* and *Rana temporaria*. However, it is possible that the pH of adult *Rana clamitans* blood is characteristically lower than that of the species tested by Kamm and Hertwig.

The relationship of the increased hydrogen-ion concentration during metamorphosis to the various degenerative changes which occur may be of several types. In the first place, it is evident that the pronounced autolytic processes operating no doubt account for an accumulation of acid metabolites in the blood stream resulting in an increase in hydrogen-ion concentration of the latter. However, it is possible that the first lowering of the pH actually precedes the onset of, and may be causative for, the early autolytic degenerations which occur in that the increased hydrogen-ion concentration either activates the autolytic enzymes or increases their rate of action in the digestion of tissue proteins. Again the tissue proteins may simply be rendered more easily digested. Regarding the above points, it should be noted that the results of the present paper (due to the necessity of killing the larvæ in order to obtain sufficient blood for the quinhydrone test) do not serve to indicate clearly whether or not a drop in pH actually precedes the first degenerative changes. It would be interesting in this connection to study the pH changes of the blood in the same individual larva immediately before and after the first degenerative tissue changes occur. This could be done with a suitable micro-electrode hydrogen-ion apparatus and without killing the larva due to the small amount of blood necessary for the test. Even if such tests should show that pH changes are purely secondary in time sequence to early degenerative changes, the probability still would remain that the heightened hydrogen-ion concentration of the blood which has been shown to occur by the results of the present paper at a relatively early stage of metamorphosis, are either causative or at least favorable to the later autolytic changes which occur during larval involution. The fact that the various organs and tissues which undergo histolysis do not degenerate all at the same stage of metamorphosis but have a definite time sequence coincident with various degrees of involution, strongly suggests a correlation between a definite blood pH and the onset of autolysis of each organ.

The writer wishes to point out the possibility that carbon dioxide escape may have affected the actual pH readings recorded for the various tests. Such escape no doubt occurred to a certain extent during the collecting and transferring of the blood samples to the electrode vessels, although the general agreement of the results obtained for any one stage of metamorphosis would seem to indicate that if such losses did occur, they were not great. Consequently, although finer micro-electrode methods coupled with suitable carbon dioxide correction factors may ultimately serve to alter the average hydrogen-ion concentrations recorded for the various metamorphic stages, it seems quite unlikely that the approximate difference of 0.3 of a pH unit between normal larval and completely metamorphosed larval blood will be seriously affected. The important point to be derived, therefore, from the present paper is not that the results strive to represent a final determination of the exact average pH typical of each stage of metamorphosis, but rather that a definite drop in pH does occur during larval involution which may be and probably is correlated with various metamorphic structural and physiological changes.

Summary and Conclusions

1. The blood pH of twenty-five normal larvæ of *Rana clamitans* was determined by means of the quinhydrone method. An average pH of 7.50 was obtained.

2. Two intermediate stages of metamorphosis were tested (twenty-five cases of each) showing an average progressive decrease in pH to 7.39 and 7.27, respectively.

3. The average blood pH of twenty-five animals, immediately following the completion of involution, amounted to 7.20. It is concluded that a definite increase in the hydrogen-ion concentration of anuran blood occurs during larval transformation amounting to an approximate drop of 0.3 of a pH unit.

4. The average blood pH of newly-metamorphosed frogs, three to four weeks after the completion of metamorphosis, was determined as approximately 7.18. It is concluded that little, if any, change in blood pH occurs within three to four weeks following the completion of larval involution, although changes may occur during future adult growth.

5. The results are discussed in general and suggestions offered regarding the relationship between the increase in hydrogen-ion concentration of the blood and the various degenerative changes which occur during larval involution.

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SEXUAL PHASES IN THE AMERICAN OYSTER (OSTREA VIRGINICA)

W. R. COE

Osborn Zoölogical Laboratory, Yale University

During the past three years there has been an accumulation of evidence which indicates that in at least three of the so-called dioecious species of the genus *Ostrea* a change of sex frequently occurs from season to season or between early life and full maturity. It may be recalled that at least ten of the more than sixty described species of the genus are regularly hermaphroditic and larviparous. Some of these exhibit a rhythmical sequence of alternating male and female phases, as Spärck (1925) and Orton (1926–27) have so fully described for *O. edulis* and as Coe (1931, 1932) has more recently shown for *O. lurida*.

Moreover, sex determination in other bivalves, as well as in some gasteropod mollusks, has long been known to be in such a labile condition that environmental changes may profoundly alter its expression. It may not be surprising, therefore, to find that changes of sex, especially protandry, as well as various aspects of intersexuality, have been found to occur in dioecious and oviparous species of oysters. For example, Roughley (1928) concluded from his observations on *O. cucullata* that that species, formerly considered dioecious, is regularly protandric, for nearly all the very small individuals were found to be males. An experiment by Amemiya (1929) has been thought to indicate that in *O. gigas* the sexual phase of each individual is determined each winter without influence from its previous sexual condition.

Many years ago Stafford (1913) found indications that the American oyster (*O. virginica*) is protandric on the Canadian coasts and presented evidence that the young animal becomes a sexually mature male when it has reached a length of about 25 mm. At 32 mm. the gonads may be distended with spermatozoa.

More recently Burkenroad (1931) has also shown that protandry occurs in this species on the coast of Louisiana. He concluded that although the sexes of the adults are morphologically separate, each individual is essentially a protandrous hermaphrodite. His evidence indicates that close association with large oysters causes some individuals to assume or retain the male phase, although other oysters of the same size, but growing singly, are predominantly females. He found small individuals to be almost always males regardless of their associations and interpreted his evidence as indicating that the likelihood of large oysters being males decreases rapidly as the distance from other individuals increases.

Further evidence of protandry in this species has been furnished by Needler (1932) from oysters collected at various localities on the North Atlantic coasts of the United States and Canada. She concludes that the majority of individuals first mature as males and that many of them later change to females. The change may be hastened by favorable nutritive conditions and may possibly be retarded by close association with older females. She observed one instance where a three-year-old male changed into a functional female in the interval preceding the next breeding season.

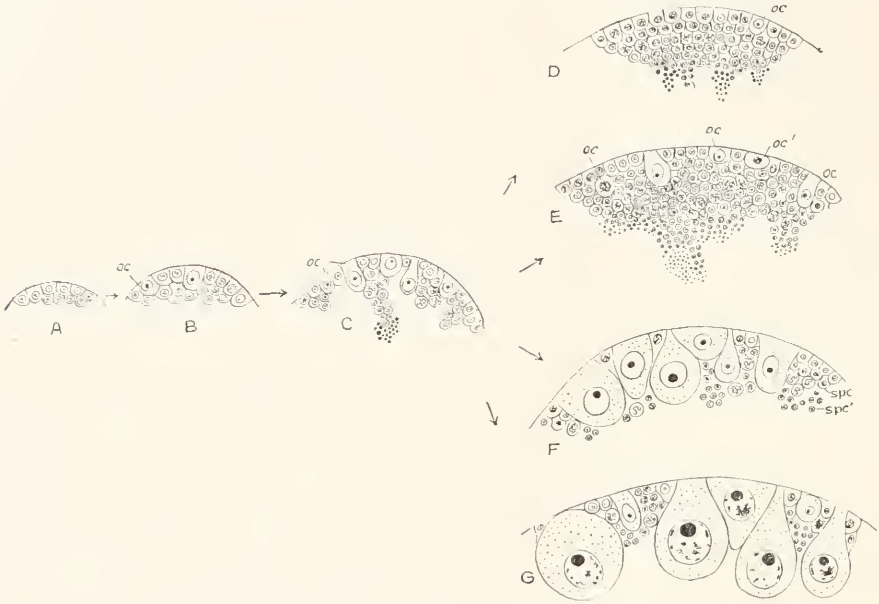

FIG. 1. Diagrams showing the primitive intersexual gonad and its transformation into the definitive spermary and ovary. *A*, early gonad with undifferentiated cells; *B*, intersexual phase, with differentiating ovocytes (*oc*); *C*, later intersexual phase with preliminary abortive spermatogenesis; *D*, spermary with only a few small ovocytes; *E*, spermary with many ovocytes, some of which (*oc'*) are in process of degeneration; *F*, young ovary, with many spermatocytes (*spc*), some of which (*spc'*) are pycnotic; *G*, nearly ripe ovary with residual cells and degenerating spermatocytes.

In order to determine more precisely the sequence of these changes in sexuality and the histological activities which accompany them, the gonads of a large number of oysters have been examined at frequent intervals during the first two years of their lives. Some of this material was collected from rocks along the shore of Long Island Sound in the vicinity of New Haven; some was taken from various natural oyster beds near Woods Hole, Massachusetts; many samples both of cultivated and untransplanted oysters from Quinnipiac River, New Haven Harbor, and Long Island Sound have been supplied by Mr. Howard W. Beach, Chairman of the Research Committee of the Oyster Growers and Dealers Association of North America, while a most instructive series of first-year stages was furnished by Mr. J. B. Glancy from the floats of the same Association at West Sayville, Great South Bay, Long Island.

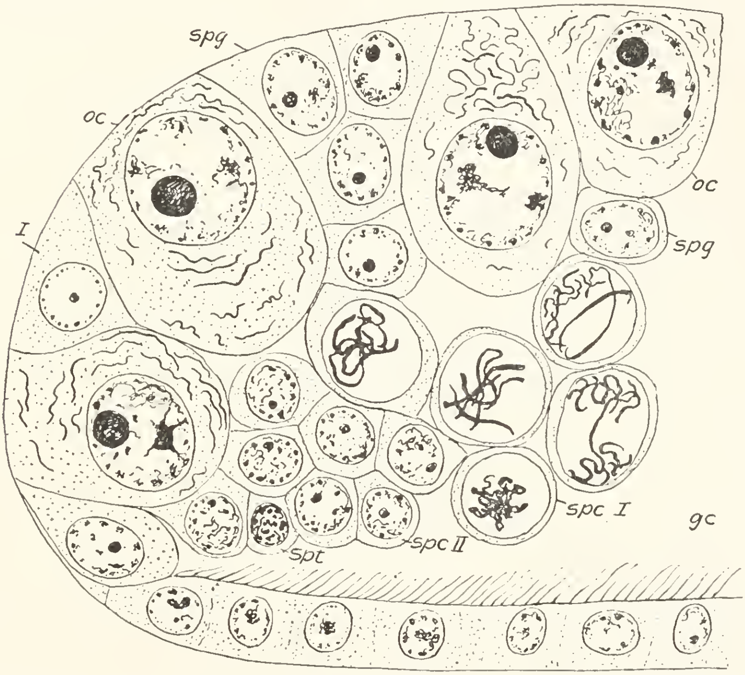

FIG. 2. Primary gonad in animal about four months of age, showing large ovocytes (*oc*), spermatogonia (*spg*), and primary spermatocytes (*spc* I) in spireme phase, with secondary spermatocytes (*spc* II) and a single spermatid (*spt*) bordering the ciliated genital canal (*gc*).

From these collections the early development of the gonads and their transformations in the course of successive phases of sexuality have been studied both in life and by means of serial sections.

Development of the Primary Bisexual Gonads

The profusely branching tubules of the primitive gonads can be found in young oysters at the age of six to eight weeks after setting or when the shell has reached a length of 6–10 mm. They ramify within the thin layer of connective tissue immediately beneath the body walls as Stafford (1913) has previously described.

In these primary gonads the germinal epithelium consists of a thin layer of morphologically undifferentiated cells which lie upon the inner side of the tubular gonad (Fig. 1), while the cells which line the outer wall, adjacent to the epidermis, become differentiated into the ciliated epithelium of the genital canals (Figs. 2, 4, 5, 6) as Hoek (1883–84) so fully described many years ago for *O. edulis* and as Coe (1932) verified in *O. lurida*.

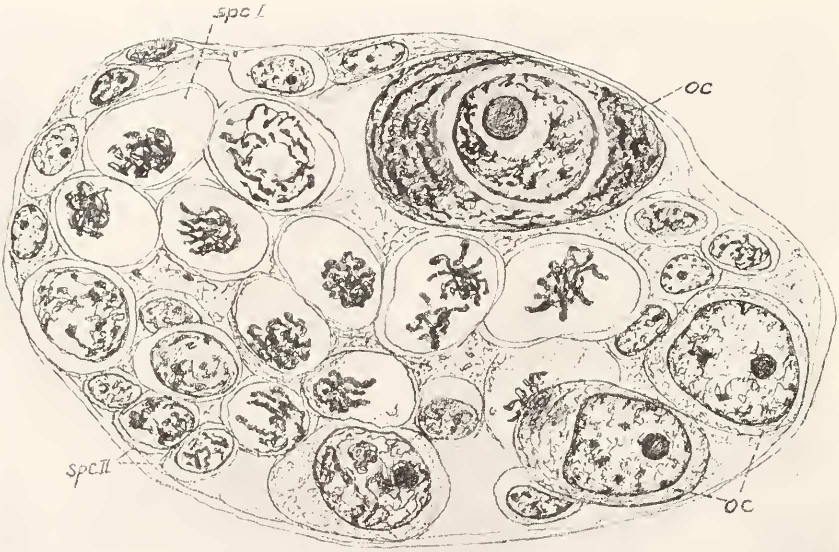

Fig. 3. Primary intersexual gonad, showing primary spermatocytes (*spc* I) and ovocytes in synapsis, secondary spermatocytes (*spc* II) and several large ovocytes (*oc*). Age about four months.

A few weeks later the germinal epithelium shows a differentiation into larger and smaller cells. The former are soon recognizable as ovocytes by the presence of fibrillar mitochondrial bodies, while many of the latter show by their rapid proliferation and later specialization that they belong to the male germ line.

By the middle of October, at the age of about three months, the shells of some individuals have become 20–25 mm. in length, and in these the gonads have already become distinctly bisexual (Figs. 2–6). A typical section through the genital canal at this age (Fig. 2) shows more or less numerous indifferent cells remaining along the inner border, interspersed with large ovocytes in which the coarse mitochondrial filaments of the so-called yolk nuclei are always conspicuous. Morphologically less well differentiated are the ovogonia and spermatogonia which resemble each other so closely that they cannot ordinarily be distinguished. Both types of cells then pass through similar synaptic phases accompanied by spiremes of coarse, densely-staining chromosomes (Figs. 2, 3, 9*B*, 9*C*) as they transform into ovocytes and primary spermatocytes respectively.

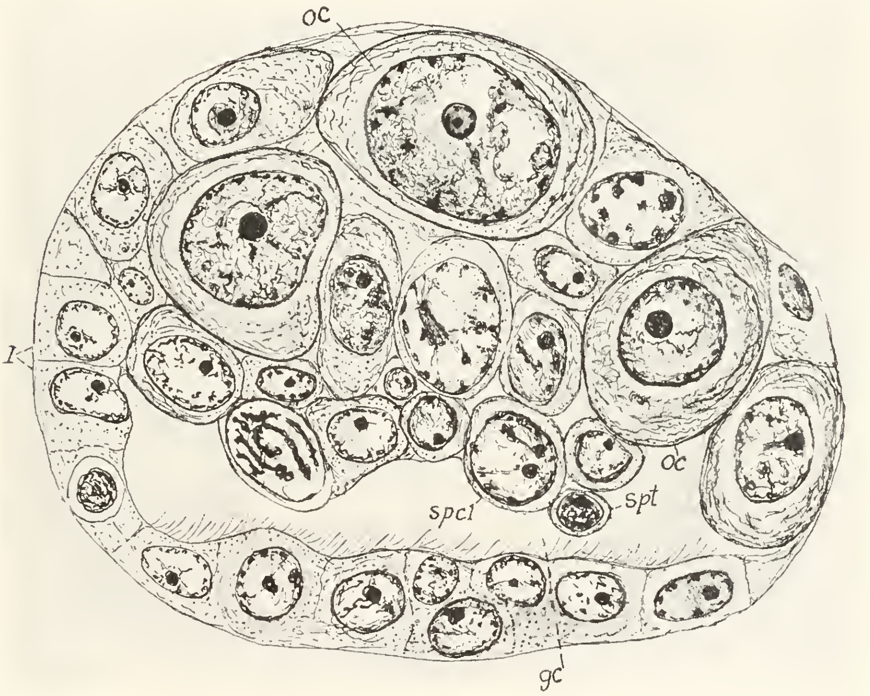

FIG. 4. Primary intersexual gonad in an animal about four months of age, showing indifferent cells (I) and several large ovocytes (*oc*), with spermatocytes (*spc* I) and a single spermatid (*spt*) bordering the ciliated genital canal (*gc*).

The spermatogonia proliferate rapidly, leading to the formation of the numerous spermatocytes which soon give the intersexual gonad a predominantly male appearance. In some individuals the spermatocytes complete their meiotic divisions to form spermatids, thereby accentuating the resemblance of the gonad to a spermary (Figs. 4–6).

In other animals of the same age the gonads may retain a closer similarity to an ovary (Figs. 2, 3), and it is improbable that any spermatids are formed in every individual. All degrees of intersexuality are found, although the vast majority of individuals are predominantly male. Not infrequently some parts of the system may assume a distinctly male appearance while adjacent follicles are characteristically female, as shown in Fig. 6.

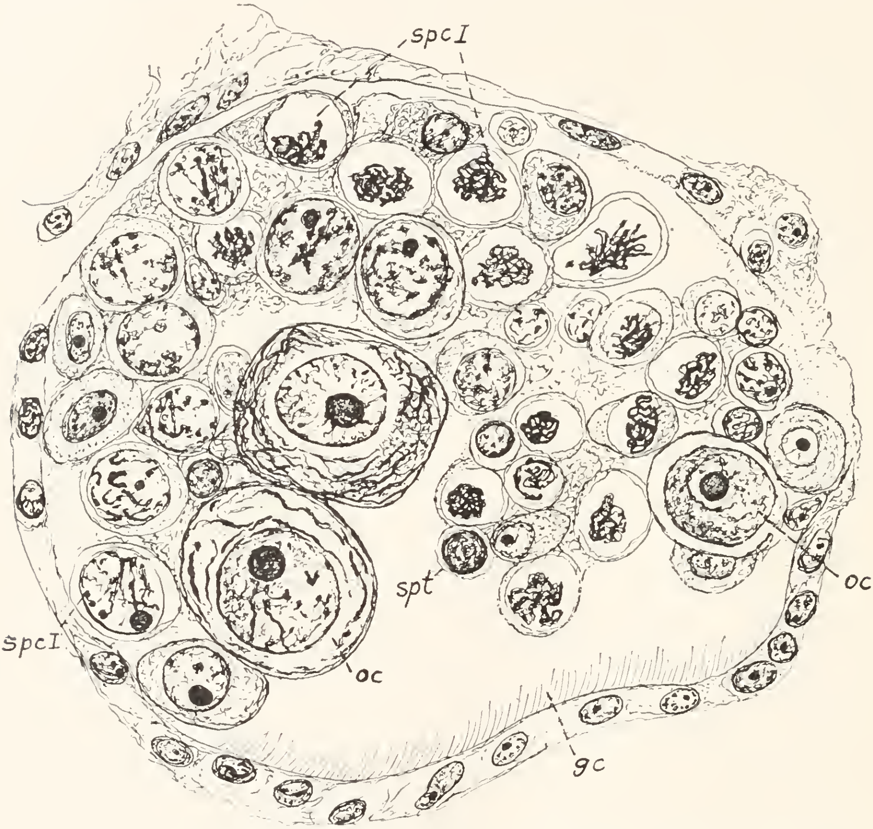

FIG. 5. Primary gonad, with ovocytes (*oc*) and primary spermatocytes (*spc* I) in synapsis; a few spermatids (*spt*) are already present on the border of the genital canal (*gc*); age about four months.

The process of spermatogenesis does not continue through the winter, however, in the localities investigated and no functional spermatozoa are formed. The gonads may continue to increase somewhat in size during December, with an increase in the numbers of secondary spermatocytes and spermatids. Their activities are then interrupted until there is a rise in the temperature of the water the following spring.

HIBERNATION

During the long period of hibernation, when the valves of the oyster remain closed, relatively minor changes occur in the primary gonads and these are mainly regressive in character. There is little, if any, increase in the size of the gonads during the oyster's first winter or in the numbers of their constituent cells. On the contrary, many of the previously differentiated cells become obviously abnormal and evidences of cytolysis are frequently seen. Remains of disintegrated cells sometimes occur in the lumens of the follicles and ducts.

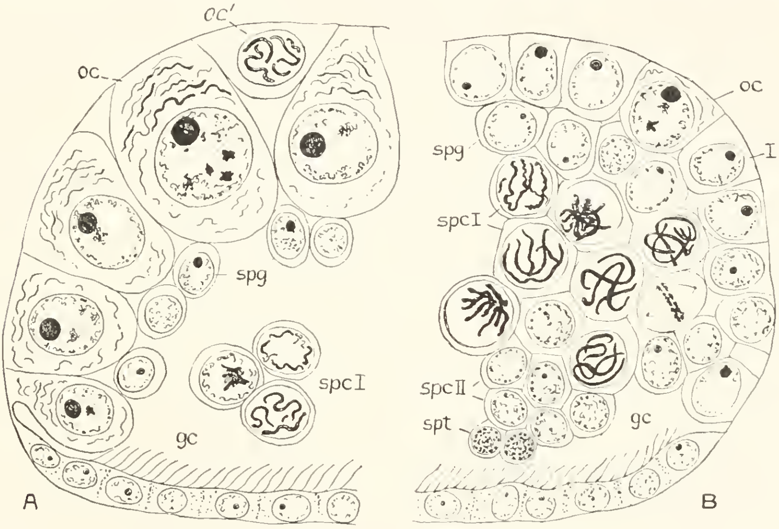

FIG. 6. Portions of two follicles from the same individual at the age of about four months: one of these (*A*) is predominantly of the female type while the other (*B*) consists mainly of spermatogenic cells, although both show some indications of their intersexual character; *oc'*, young ovocyte in spireme phase; other letters as in Fig. 2.

TRANSFORMATION TO FUNCTIONAL GONADS

At West Sayville the animal retains its primary or immature gonad throughout the winter as the figures in Table I will show. As the water becomes warmer, however, spermatogenesis is resumed in some of the larger animals and in March about one-fourth of the young oysters examined had transformed into males. No females were recognized until a month later.

In an occasional individual, due apparently to poor nutrition, the primary bisexual type of gonad is retained until the second year, at which time a few functional spermatozoa may be formed.

Males

The transformation of the bisexual gonad into a spermary consists in the proliferation of spermatogonia and spermatocytes, usually accompanied by the disintegration of some of the previously formed ovocytes (Fig. 1). There is a rapid extension of the branching system of follicles, the new branches being, as a rule, exclusively male in appearance, for the reason that the ovocytes usually found in the older parts of the system are not carried into the new follicles (Fig. 8). But this rule is not without exceptions, for many grades of intersexuality occur and the gonad is classed as a spermary in this report if it has a distinct preponderance of spermatogenic cells, even though many ovocytes are situated along the walls of the follicles (Fig. 12, *A*). The term hermaphrodite is reserved for those cases in which there are extensive areas or large masses of the cells representing each of the sexes (Fig. 12, *B*).

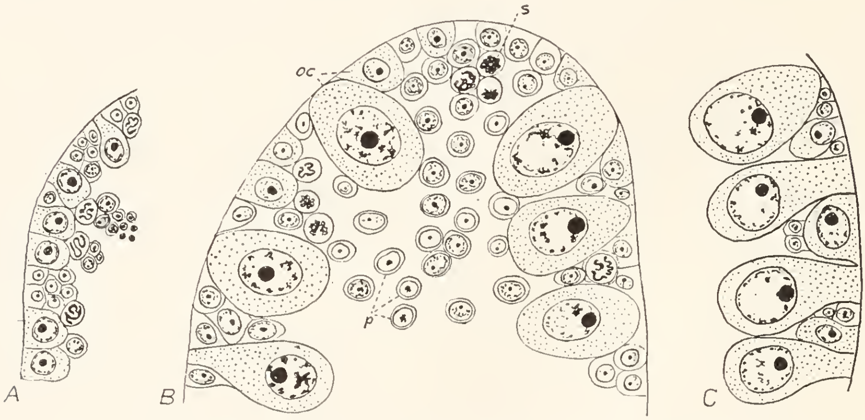

FIG. 7. Transition stages in the development of the mature ovary (*C*) from the primitive intersexual gonad (*A*). *B*, developing ovary in an individual about ten months of age, showing numerous small pycnotic cells (*p*) in the lumen and between the ovocytes (*oc*); *s*, cells in spireme phase.

Females

As the season advances an increasing proportion of the young oysters attain sexual maturity, as shown in Table I. During April

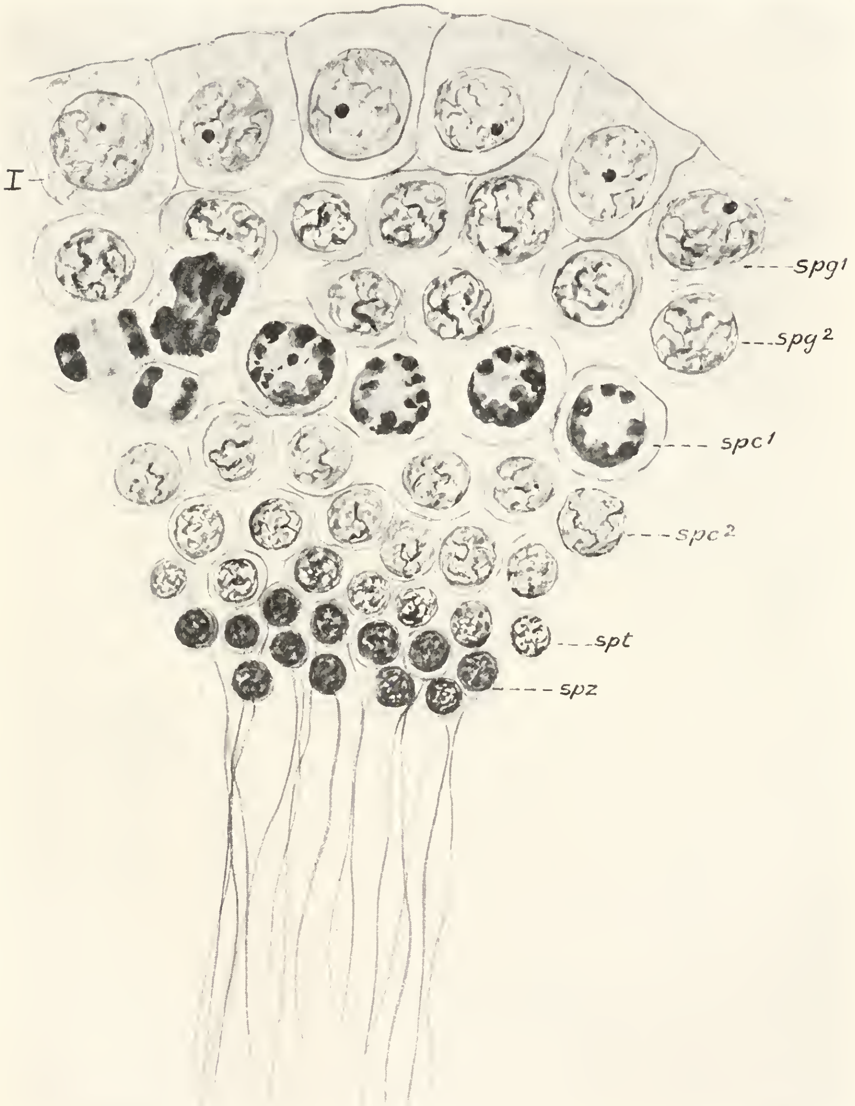

FIG. 8. Mature spermary, showing indifferent residual cells (*I*) and normal spermatogenesis; *spg*[1], *spg*[2], primary and secondary spermatogonia; *spc*[1], *spc*[2], primary and secondary spermatocytes; *spt*, spermatids; *spz*, mature spermatozoa.

the first females were found and these showed the transition of the intersexual gland into an ovary (Fig. 7). This process is accomplished by the concurrent growth of the primitive ovocytes, with additions from the small ovogonia, and the pycnosis and eventual cytolysis of such secondary spermatocytes and spermatids as may be present (Fig. 7).

Many indifferent cells and presumably some of the spermatogonia remain as residual cells even after ovulation has occurred. The first crop of eggs discharged is very small, as only a minute proportion of the definitive ovocytes reach maturity until after the first ovulation (Fig. 9). The genesis of the ripe ovum, measuring about .05 mm. in diameter has been so fully described that further discussion here is unnecessary. The spawning reactions of both male and female have been fully investigated in this and other species by Prytherch (1924) and by Galtsoff (1932).

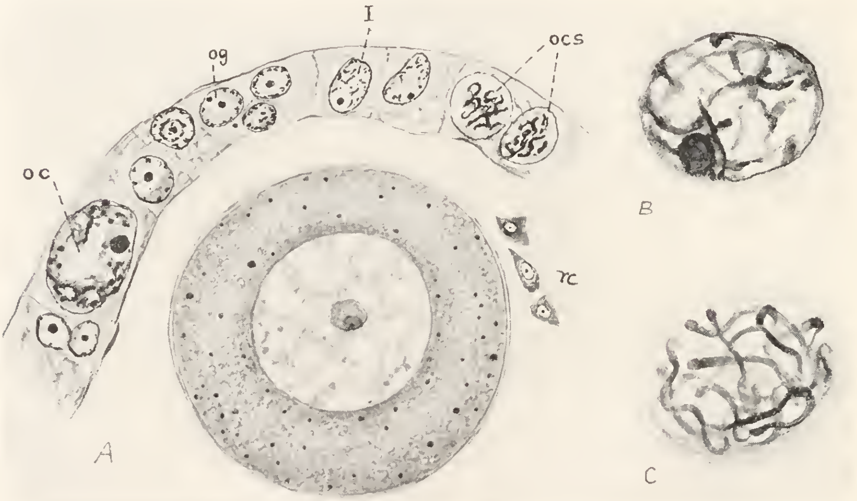

FIG. 9. *A*, mature ovary, showing a single ripe ovum and the residual cells of the follicle; *I*, indifferent residual cells; *og*, ovogonia; *ocs*, young ovocytes in synaptic phase; *oc*, residual ovocyte; several pycnotic residual cells (*rc*) are free in the lumen. *B*, *C*, spireme stages in young ovocytes.

Functional Hermaphrodites

True hermaphroditism in the adult American oyster had been considered a rarity until Burkenroad (1931) reported that about 1 per cent of the general population on the coast of Louisiana belonged in this category. Needler (1932) also found about the same percentage of hermaphrodites from one locality and a smaller proportion from other areas on the coast of Prince Edward Island. Those that she found were in their third year except one each in their fourth and fifth years.

Although the primary gonad is normally bisexual and at least some degree of intersexuality is usually found in sexually mature yearlings, only 4 among 96 such individuals from W. Sayville and 4 among 389 from New Haven Harbor were classed as true hermaphrodites. These showed approximately equal areas of male and female cells (Fig. 12, *B*). Several others were considered to be hermaphroditic males, being provided with spermaries of normal appearance except for the presence of scattered ovocytes (Fig. 12, *A*), sometimes fully grown.

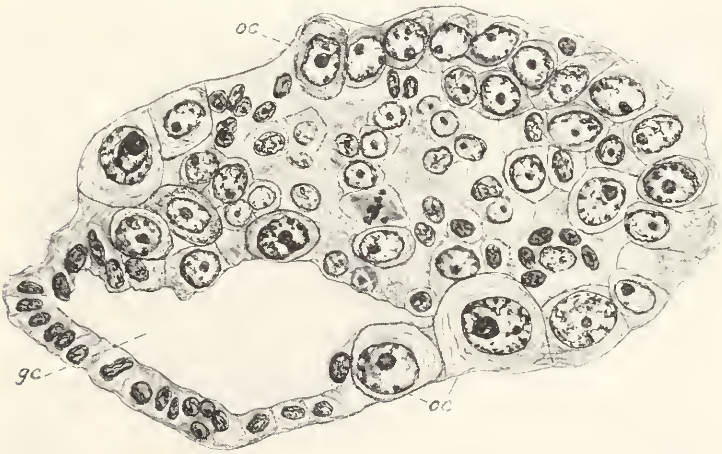

FIG. 10. Spermary early in second year, showing its continued intersexual character; *gc*, genital canal; *oc*, ovocytes.

SELF-FERTILIZATION

Among 55 sexually mature yearlings taken from the culture floats at West Sayville on June 23 were two functional hermaphrodites. Many of the ova in each of these proved capable of self-fertilization and apparently normal development in spite of the vast excess of sperm present. Needler (1932) has reported a similar observation.

SPERMATOGENESIS

The successive stages in the process of spermatogenesis are similar to those described by Coe (1932) for *O. lurida* except that the spermato-

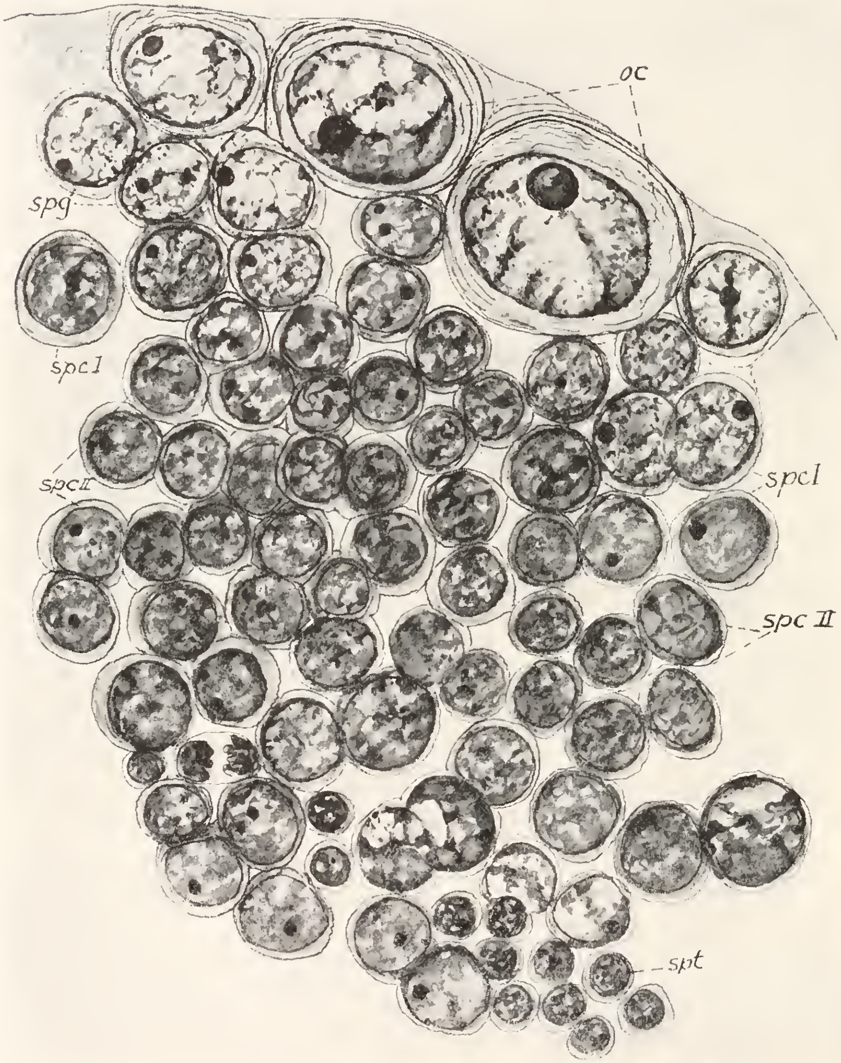
oc
spg
spc I
spc II
spc I
spc II
spt

FIG. 11. Spermary at end of second year, showing its continued intersexuality by the presence of large ovocytes (*oc*) on wall of follicle; letters as in Fig. 2.

cytes and spermatids are free to separate immediately after their formation (Fig. 8), instead of adhering in masses as they do in the latter species where the spermatozoa are united into sperm-balls.

Gonads at the End of the Breeding Season and During the Second Year

Such individuals as become sexually mature during their first year produce but a relatively small number of gametes, retaining as residual cells a large proportion of the germinal cells composing the gonads. The residual germinal epithelium of the ovary is similar to that shown in Fig. 9, *A* (except for the absence of the single ripe ovum), while Fig. 10 shows a section of a spermary shortly after activity has been resumed near the beginning of the second year.

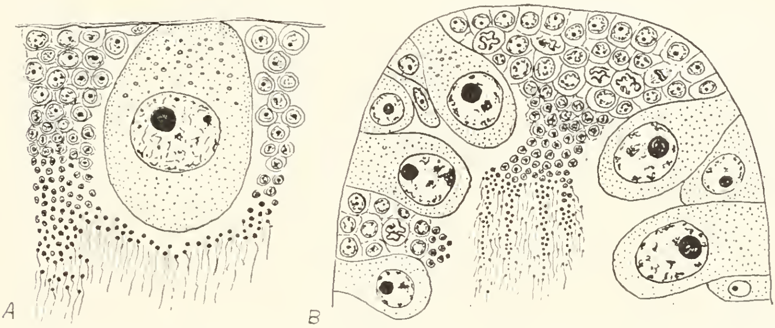

Fig. 12. *A*, partial hermaphroditism; portion of spermary of hermaphroditic male, showing one of the scattered ovocytes. *B*, complete hermaphroditism; gonad with about equal areas of ovarian and spermatogenic tissue. Age about ten months.

The gonads of both sexes after spawning usually retain indications of their continued intersexual character; in the males by the presence of ovocytes along the walls (Figs. 10, 11), and in the females by groups of small cells similar to those shown in Fig. 7. In the latter case, however, there is as yet no proof that these small cells actually belong to the male line, for active spermatogenesis has not been found in an individual classed as a female. A spermary may have few or many ovocytes which sometimes produce yolk in a normal manner, but the reverse conditions seem not to hold except in the relatively small proportion of individuals which exhibit functional hermaphroditism.

Sex Reversal

The observations of both Burkenroad (1931) and Needler (1932) indicate a strong tendency toward protandry in this species. The former concluded that the change to the female phase takes place when the shell of the young animal has reached a length of about 40 mm., while Needler has positively proved that a change of sex may occur as late as the third or fourth year.

It has hitherto been an open question whether all individuals are protandric; that is, whether the relatively few oysters which spawn as females at the end of their first year may have passed through a preliminary male phase the preceding autumn. It can now be answered that such is not the case in the localities under consideration. The ovary of yearling females develops directly out of the primary intersexual gonad by the growth of the primordial ovocytes and the elimination of spermatocytes and spermatids before spermatogenesis has been completed. The preliminary male phase is thus abortive in these localities although such may not be the case in the warmer areas farther south along the coast.

CORRELATION OF AGE, SIZE, AND SEX DURING THE FIRST YEAR

It has been stated above that at West Sayville, Long Island, a few of the more rapidly growing young oysters become sexually mature as males during March of their first year. Females were first recognized in April and these were mainly among the largest individuals of the group.

Tables I and III show the numbers of individuals of each sex and of each size found at three different periods preceding the breeding season and once after spawning had commenced. The average size of the young females always exceeds that of the sexually mature males of the same age if considerable numbers of each are considered. This rule is shown by Needler (1932) to hold also for oysters at the age of two and of three years, but in still older animals the males are said to average as large as those of the other sex.

The greater size of the females of the younger ages may be correlated (*a*) with a more efficient metabolism associated with the female sex mechanism, or (*b*) greater activity of the female in obtaining food, or (*c*) the actual differentiation of the individual into a female as the result either of its inherent metabolic potentialities or its favorable environmental conditions, or both.

And, conversely, the responsible agency for the determination of maleness and slower growth may be a less favorable metabolism, either genetic or environmental, or, conceivably, the retarding influence of older, associated individuals of either sex.

Inspection of Table I shows clearly that the collection made on March 23 represents a large proportion of oysters that would have become sexually mature later in the season and these would presumably have included both sexes. Omitting this collection and combining the two groups taken April 29 and May 21 shows (Table II) that shortly before the beginning of the breeding season in June, 41.6 per cent of 149 specimens sent for examination were still immature, while 41 per cent were males, 1.3 per cent hermaphroditic, and 16 per cent were females. By a curious coincidence these figures agree surprisingly closely with those obtained by Needler (1932) from the same locality at the end of June, 1931. She found 45.3 per cent immature, 38.7 per cent males, and 16 per cent females among 119 individuals which she examined.

Table I

Correlation of Size and Sex during First Year, W. Sayville

Length	March 23			April 29			May 21				June 23			
	Im.	M.	F.	Im.	M.	F.	Im.	M.	H.	F.	Im.	M.	H.	F.
mm.														
10–19 . .	6	—	—	16	—	—	4	—	—	—	2	—	—	—
20–29 . .	6	—	—	19	1	—	8	—	—	—	5	3	—	—
30–39 . .	7	1	—	4	12	2	9	14	—	1	2	8	—	—
40–49 . .	7	3	—	1	12	1	1	7	—	1	—	11	1	1
50–59 . .	—	2	—	—	5	10	—	7	2	4	—	7	—	7
60–69 . .	—	1	—	—	—	3	—	3	—	2	—	12	1	1
70–79 . .	—	—	—	—	—	—	—	—	—	—	—	—	—	3
Total. . .	26	7	0	40	30	16	22	31	2	8	9	41	2	12

Table II

Correlation of Size and Sex; W. Sayville. Groups from April 29, May 21, and June 23 combined.

	Total	Im.	p.c.	M.	p.c.	H.	p.c.	F.	p.c.
Less than 40 mm.	110	69	62.7	38	34.5	0	0	3	2.7
More than 40 mm.	103	2	2.0	64	62.1	4	4.0	33	32.0
Less than 50 mm.	146	71	48.6	68	46.6	1	0.9	6	4.1
More than 50 mm.	67	0	0	34	50.7	3	4.5	30	44.8
Total.	213	71	33.3	102	47.9	4	2.0	36	17.0

Im., immature; sex not determinable; M., male; H., hermaphrodite; F., female.

On June 23, 1932, however, the writer found that only 14 per cent of the 64 samples studied were still immature, while 64 per cent were males, 3 per cent functional hermaphrodites, and 19 per cent females

(Table I). The smaller proportion of sexually mature yearlings in 1931 as compared with 1932 may be accounted for by the somewhat higher temperature of the water during the growing season of the latter year.

Combining the three groups examined April 29, May 21, and June 23, 1932, numbering 213 individuals, shows 71, or 3.33 per cent, immature; 102, or 47.9 per cent, males; 4, or 2 per cent, functional hermaphrodites; and 36, or 17 per cent, females (Table II).

Considering only the 149 individuals of the present collections that were sexually differentiated we find that they comprise 109 males; 4 hermaphrodites, and 36 females, a percentage ratio of 73.2, 2.7, and 24.2 respectively.

TABLE III

Correlation of Size Groups and Sex First Year; W. Sayville

Length	March 23			April 29			May 21				June 23			
	Im.	M.	F.	Im.	M.	F.	Im.	M.	H.	F.	Im.	M.	H.	F.
mm. Less than 40	19	1	0	39	13	2	21	14	0	1	9	11	0	0
More than 40	7	6	0	1	17	14	1	17	2	7	0	30	2	12
Less than 50	26	4	0	40	25	3	22	21	0	2	9	22	1	1
More than 50	0	3	0	0	5	13	0	10	2	6	0	19	1	11
Total	33			86			63				64			

Im., immature; sex not determinable; M. male; H. hermaphrodite; F. female.

At the end of June, 1931, Needler found 46 males and 19 females among the 65 sexually mature individuals which she examined from the same locality. Combining these figures with those of Table II gives a total of 155 males, 4 hermaphrodites, and 55 females, a ratio of nearly 3 males to 1 female.

Of the sexually mature individuals less than 40 mm. in length (Table III) there were 39 males to 3 females, but those of larger size showed 70 males and 33 females. But in the class measuring more than 50 mm. in length (Table III) there are nearly as many females as males, indicating a rather definite correlation of size and sex, as Burkenroad (1931) has shown for the general population and Needler (1932) for the age groups. Probably the most reliable correlation of sex and size is shown by the collection made during the early part of the breeding season (June 23), representing as nearly as could be estimated an average sample of the entire population remaining on the floats. None of the 12 females was then less than 40 mm. in length and only one of them less than 50 mm. Approximately 27 per cent of the 41 males, on the other hand, measured less than 40 mm. and 54 per cent less than 50 mm. Those in the largest-sized group (70–79 mm.) were all females (Table I). Near the end of the spawning season (July 29) a collection of 70 individuals showed a sex ratio of 100 males to 23 females. Of those measuring less than 50 mm. in length there were 37 males but no females, while those of larger size comprised 15 males and 12 females. Only a single individual was still immature.

The correlation of sex and size is actually much closer than the tables indicate, for the figures given are based upon the length of the shell alone and not upon the actual size of the animal. Selection based on volume would undoubtedly give a still larger percentage of females at the end of the first year in the locality under consideration.

In other areas, however, the sex ratios at the end of the first year are quite different, as Needler (1932) has shown by comparing one-year-old oysters from Prince Edward Island, Long Island Sound, and Great South Bay, Long Island. She concluded that in the colder areas, with a shorter season of activity, relatively few individuals became sexually mature as males before their second year and none as females.

First-year Oysters from Quinnipiac River and New Haven Harbor

Collections were made monthly from September, 1931, to July, 1932, and a very large number of young oysters were examined. Nearly a hundred of these were cut in serial sections. Conditions were unfavorable for a large set in these areas during the summer of 1931 and there was a high mortality of the young oysters in exposed situations during the ensuing winter.

In November, when about four months of age, the shells of the largest individuals were from 20 to 27 mm. in length, but the majority measured only 5 to 15 mm. The gonads of the smaller individuals were beginning to branch out beneath the body walls, while those of the larger animals had already reached the primary bisexual phase, with differentiated ovocytes and spermatocytes (Figs. 2–6). A few spermatids were also present in some individuals.

The rate of growth and the accompanying sexual changes through the ensuing months and until the breeding season in July are summarized in Table IV. The table shows that the first individuals to become recognizably differentiated sexually were all males, and none of these could be identified with certainty until April or May. During June there was a relatively rapid growth in size, accompanied by an increasing number of sexually differentiated males among the larger individuals. But the correlation of size with sexual differentiation is not without exceptions, as Tables I and IV will show, although the sexual conditions may be definitely controlled by nutrition. Spermatogenesis or ovogenesis may be inaugurated when nutritive conditions are favorable but growth may then be checked by the encroachment of other individuals, while gametogenesis continues. The result may be a dwarfed but sexually functional animal.

TABLE IV

Correlation of Age, Size and Sex, Quinnipiac River and New Haven Harbor

Length	Nov.	Dec.	Mar.	May			July				
	Im.	Im.	Im.	Im.	M.	F.	Im.	M.	H.	F.	Total
mm.											
5– 9......	Many	Many	Many	Many	0	0	0	0	0	0	0
10–14......	Many	Many	Many	Many	0	0	2	0	0	0	2
15–19......	Some	Many	Many	Many	0	0	6	11	0	0	17
20–24......	Few	Some	Some	Some	Few	0	9	41	0	0	50
25–29......	Few	Few	Few	Few	Few	0	0	104	1	2	107
30–34......	0	0	0	Few	Few	0	0	116	1	3	120
35–39......	0	0	0	0	0	0	0	82	2	2	86
40–44......	0	0	0	0	0	0	0	26	0	3	29
45–49......	0	0	0	0	0	0	0	7	0	1	8
50–54......	0	0	0	0	0	0	0	2	0	2	4
Total....	—	—	—	—	Few	0	17	389	4	13	423

An examination of 423 yearlings taken at random at the height of the breeding season in July indicated that about 96 per cent of the survivors from the set of the previous year had become sexually mature. Most of these were males filled with spermatocytes and ripe spermatozoa (Table IV). The other 4 per cent still retained the primary intersexual gonads and indicated that sexual maturity had been postponed until their second year. All of these immature individuals, as would be expected, showed evidence of unfavorable nutritive conditions as indicated by their dwarfed size.

The predominantly protandric nature of the group is shown by the fact that 389, or nearly 96 per cent of the 406 sexually mature individuals examined were more or less fully ripe males, while only 13, or 3.2 per cent, were females. Four, or 1 per cent, were functional hermaphrodites with large masses of both ova and spermatozoa. The ratio of males to females is thus 100 : 3.3.

While the number of females is too small to be statistically dependable, it will be observed that their mean length is somewhat greater than that of the males, but less conspicuously so than in the much larger group from West Sayville (Table I). If the volumes had been measured, instead of the lengths, the females would have shown a still greater comparative size.

Comparison of Tables I and IV will show that the proportion of individuals which mature as females during their first year at West Sayville and in New Haven Harbor is about ten times as great in the former locality as in the latter. The size differences of the two populations of yearlings show that not only is there a correlation between rate of growth and sex as concerns individuals but that the proportion of females is several times higher in the locality where the conditions for growth are the more favorable or the growing season longer. There may be some question as to whether the evidence is sufficient to justify the conclusion that metabolic conditions at the time of sexual differentiation actually determine the direction taken by the primary intersexual gonad in its transformation into the functional organ of the first sexual phase. But until further experimental evidence is available it seems to be the most reasonable hypothesis suggested.

First Year Oysters from Near Woods Hole, Massachusetts

Young oysters from small natural beds in the vicinity of Great Harbor, from the shores of the neighboring islands, and from Onset were examined in the summers of 1931 and 1932. In all of these areas growth is slow during the first year and the shell seldom reaches a length exceeding 30 to 35 mm. The more usual length is 6 to 20 mm.

An examination of 389 yearling oysters from these localities showed that 373, or nearly 96 per cent, were still immature or the sex undeterminable, 9 were males, 3 were hermaphrodites and 4 were females. At the age of two years, when most of the young oysters in that region become sexually mature, the ratio of males to females is about one hundred to fifty-five, with approximately two per cent true hermaphrodites. At this age the average size of the females considerably exceeds that of the males, as was also the case in the other areas during the first breeding season.

Comparison with Other Species

The primary bisexuality of *O. virginica* and the development of the definitive gonads, including later sex reversals, are in many respects similar to the series of sexual phases which characterize such strictly hermaphroditic and larviparous species as *O. edulis* (Orton, 1926–27) and *O. lurida* (Coe, 1931, 1932). In both the hermaphroditic and seasonally dioecious types the more rapid proliferation of spermatogonia as compared with the ovocytes soon gives the early gonad its predominantly male characteristics. In the former type, however, all individuals are thought to become functional males before assuming the female phase, while in *O. virginica* from 3 to 30 per cent of the sexually mature young individuals at different localities show only an abortive male phase, the primary gonad developing into an ovary without completed spermatogenesis.

In each type residual cells of both sex lines remain after the first spawning, providing a cellular mechanism which leads in some of the hermaphroditic forms to a series of alternating sexual phases, while in *O. virginica* the sexual changes appear to be more or less facultative, for it is known that in at least some individuals the same sexual phase may be retained for several years. This is presumably true of the great majority of adult oysters under a stable environment.

Evidences of protandry and sex change have been reported in other species of oviparous oysters. Of a large number of very young *O. cucullata* examined by Roughley (1928) all except about 5 per cent were males and he suspected that these exceptions might previously have spawned as males. He found nine functional hermaphrodites. These were thought to represent stages in the transformation of male to female, but it seems more probable that the sexual conditions in that species are not very different from those here described for *O. virginica*, and that the change of sexuality in both species takes place in the interval between two breeding seasons. Among 3000 large adult oysters from thirty different localities he found a sex ratio of 270 females to 100 males.

In *O. angulata* also hermaphroditism occurs occasionally. Amemiya (1925) found two such individuals among 14 males and 59 females. But it is not known whether that species, which is closely allied to *O. virginica*, experiences similar sex changes.

A most interesting type of sex change has been recently reported by Amemiya (1929) for the Japanese oyster (*O. gigas*), previously considered dioecious. In one summer a small hole was made in the shell of each of several hundred oysters and the sex thereby determined. The sexes were then placed in separate cages and returned to the sea. A year later it was found that oysters of both sexes were present in each cage. It was concluded that about 25 per cent of the females and 60 per cent of the males had changed their sex during the winter. If these conclusions prove to be well founded, Amemiya's hypothesis, that the sex of any individual of that species is determined each winter independently of its previous sexual conditions, will add a new phase to the many variants of sexuality in animals.

Since there is such a wide diversity in the abundance and size of the ovocytes in the sexually mature young males of *O. virginica*, it is pertinent to inquire whether there may not be two genetically distinct types of these males. The samples studied indicate, as shown in Table II, that at West Sayville about 48 per cent of the entire one-year group or more than 70 per cent of the sexually mature yearlings function as males, while in New Haven Harbor there are fully thirty males to one female at their first breeding season (Table IV). It is conceivable that those males with but few and very small ovocytes are genetically "true males" while those with more numerous and larger ovocytes may represent the protandric males. Perhaps it is only the latter that later undergo sex reversal, as Orton (1928) has suggested for *Patella*. It would probably be unwise to speculate further in this connection until more complete evidence is available. The appearance of the primary gonads, however, and the changes which they subsequently undergo, suggest that sexuality in this species may rest upon a basis somewhat comparable with that which Witschi (1932) has found in certain races of frogs.

SUMMARY

1. Examination of the developing gonads of young oysters from various localities at frequent intervals during the first two years of life shows that a primary bisexual gonad is formed in each individual within a few months after setting.

2. The activities of the gonad depend upon the temperature of the water and apparently other conditions of nutrition, a much larger proportion of the animals becoming sexually mature during the first year in warmer than in cooler localities.

3. The primary gonad contains the antecedent cells of both sexes, with ovocytes upon the walls of the follicles and spermatocytes intermingled and bordering the lumens.

4. The protandric nature of the primary gonad frequently becomes manifest by the rapid proliferation of the spermatogonia and the formation of primary spermatocytes; the latter soon pass through the synaptic phases and lead to the production of secondary spermatocytes

and spermatids at the age of a few months. But no functional spermatozoa have been observed until the following spring in the areas investigated. The species is not strictly protandric, however, for 3 to 30 per cent of the sexually mature yearlings are females, the ovaries of which have developed directly from the primary gonads without the completion of a preliminary functional male phase.

5. The definitive sexual gland is a transformation of the primary gonad by the proliferation of spermatogonia and the disintegration of many of the ovocytes to form the spermary or, less frequently, the growth of ovocytes, accompanied by the disintegration of spermatocytes and such spermatids as may be present, to form an ovary. But the intersexual character is usually retained to at least some extent in both types of gonads.

6. The proportion of male and female cells in the mature gonad is highly variable, a few large ovocytes being frequently found in some parts of otherwise typical spermaries, while in the ovary some follicles may retain characteristic male cells. True hermaphroditism was found in 1 to 4 per cent of the sexually mature oysters at the end of their first year. Apparently normal development follows self-fertilization.

7. In the warmer of the two principal localities investigated about 70 to 80 per cent of the oysters which became sexually mature during their first year were males. In the cooler locality the proportion of males exceeded 95 per cent.

8. Most of the relatively small number of females are among the largest of their age group, indicating a close correlation between sex and size. This may imply that the female is metabolically the more active sex or that she requires better nutritive conditions in order to mature, or that sex in this species is so labile that the nutritive conditions of the individual at the critical period of sex differentiation determine which of the alternative types of cells in the primary bisexual gonad shall predominate. Alternative genetical explanations are discussed.

9. After the animal has spawned as male or female the gonad may still retain its bisexual character; the sexual phase during the following year may again depend largely upon nutritive conditions.

10. The primary bisexuality of this species and the cellular mechanism for sex reversal here reported are interpreted with reference to related species of the genus in which hermaphroditism and alternating sexual phases have been retained.

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A COMPARISON OF THE LIFE HISTORIES OF MICTIC AND AMICTIC FEMALES IN THE ROTIFER, HYDATINA SENTA[1]

JOSEPHINE CAROLYN FERRIS

Department of Zoölogy, University of Nebraska

Introduction

The life history of the rotifer, *Hydatina senta*, involves the reproduction of two kinds of females, amictic females, those which reproduce wholly by parthenogenesis, and mictic females which reproduce parthenogenetically or bisexually.

A comparison of these two types of females in regard to the periods of their life histories was made in an endeavor to find if there is a difference in the metabolic rate of the two and to discover its possible relation to the factors which regulate the production of these two kinds of females.

Miller found that the three types of individuals, amictic females, mictic females, and males of *Lecane inermis* differed in the length of the total life period. The unfertilized mictic females and amictic females not only differ in length of life but also differ in rate of production, duration of fecund and post-fecund periods, and in the degree of correlation between fecundity and length of life.

Miller states that the difference in length of life of these two kinds of females is due to the fact that egg-production is a less strenuous process in mictic females than in the amictic ones and consequently the mictic females survive the fecund period and pass entirely through the post-fecund, dying a natural death in old age. The mictic females produce fewer and smaller eggs than the amictic females at a slower rate and they cease egg-production at an earlier age. The differences in length of life would be due probably to the differences in the metabolic rate in the fecund period.

Jennings and Lynch point out that in the case of *Proales sordida* there is no correlation between length of life and the number of eggs produced in the amictic females but that diversities in fecundity were due rather to the size of the eggs from which they have hatched. Smaller eggs which are supposed to have been produced early in the family produce less fecund individual daughters than the larger eggs produced later.

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[1] Studies from the Zoölogical Laboratory, University of Nebraska, No. 172.

Like *Proales sordida* and *Lecane inermis*, there are four distinct periods which can be distinguished in the history of the individual in *Hydatina senta:* (1) the hatching or embryonic period; (2) the prefecund or adolescent period of rapid growth; (3) the fecund or egg-laying period; (4) the post-fecund or old age period.

Hydatina senta is one of the larger rotifers commonly found in stagnant and foul ponds. It has been worked with a great deal in laboratories because it is easily cultivated, hardy, multiplies rapidly and has sexual and parthenogenetic generations. The females used in the present work were taken from a general culture which had been collected from a goldfish pool at Seward, Nebraska, in May 1931.

The experiments and observations were made at the suggestion and under the supervision of Professor D. D. Whitney to whom the author wishes to express her indebtedness for advice and assistance given.

MATERIALS AND CULTURE METHODS

Hydatina senta is easily cultured in a variety of solutions. A very favorable one is made by using old hay tea as a basis. This is prepared by boiling 1 gram of ground timothy hay in 4000 cc. of tap water for 10 minutes. It is then strained and allowed to age for 4–6 weeks before using. The tap water from which it was made was placed in direct sunlight for several hours to remove an objectionable amount of chlorine. To 100 cc. of this aged hay tea there was added 1 cc. of 1 per cent urea solution, 1 cc. of 1 per cent ox-gall solution and 1 cc. blood solution. This combination of ingredients made a very favorable culture medium for these rotifers.

The urea stock solution consisted of 1 gram of urea crystals dissolved in 100 cc. of tap water and brought to a boil; the ox-gall solution was prepared by using 1 gram of dried ox-gall plus 100 cc. of tap water and brought to a boil. The blood solution was prepared by using 1 gram of dried blood plus 100 cc. of tap water, brought to a boil and filtered.

Throughout the experiment a pure culture of the flagellate, *Polytoma*, was used as the food. This was prepared by using 1200 cc. of tap water that had been boiled, cooled, and put into a small battery jar. Into this was placed a muslin bag containing 200 grams of bone meal which previously had been brought to a boil and allowed to cool. A fresh hay tea solution also was added which was prepared by boiling for 10 minutes 1 gram of ground timothy hay in 100 cc. of sunned water. This culture was inoculated with *Polytoma* and placed at room temperature in a north light exposure. To maintain a good culture of *Polytoma*, the bag of bone meal was changed every 48 hours and fresh hay tea solution added. After a few days, however, the culture water would become too foul and develop a red coating of bacterial growth on the walls of the jar. Whenever this occurred a new culture was made by pouring the top of the old culture into another sterilized jar and adding enough sterilized water to make 1200 cc. Then fresh bone meal and fresh hay tea solution were added as stated above. In this manner a vigorous culture of *Polytoma* was maintained for many months.

Food for the rotifers from this culture was prepared daily by removing the film from the surface with a sterilized spoon and thus obtaining *Polytoma* in countless numbers. These were then washed twice with old hay tea solution by means of the centrifuge. One cubic centimeter of this concentrated *Polytoma* was diluted with 15 cc. of old hay tea solution. One drop of this was then placed daily in each individual watch-glass containing one female in 7 cc. of culture solution.

The life histories of 184 amictic, 113 mictic females, and 88 mictic females whose eggs had been fertilized were studied and compared.

The females were all cultivated under similar conditions and kept at a constant temperature of 16° to 17° C. This temperature was maintained by use of a double-walled temperature bath through which there was a continual in- and out-flow of tap water. During the winter months the temperature of this bath was kept quite constant and the entire observations were made during this period.

The record for the life history periods began at the time of isolation of the eggs. This was done by isolating in a container a group of mothers that were about ready to lay eggs. They were given a great deal of food and then observed every hour. The first lot of eggs was not recorded due to the fact that some of them may have been laid previously and have been isolated with the mothers. However, beginning at the end of the first hour after they had begun to lay, the eggs were isolated every hour, placed in a container, and labelled. In this way it was known that the eggs isolated at any particular time had been produced during the preceding hour. These eggs were all placed in the temperature bath and carefully observed on the following day for the hatching of the young females. Upon hatching, the young females were immediately isolated and each placed in a separate Syracuse watch-glass with fresh culture solution. Observations were made twice a day on these young females and an effort was made to obtain within a few hours the hatching-time of the first offspring of each female. Thereafter throughout the experiment each individual was looked at twice daily and observations made.

During the fecund period the offspring of each female were removed twice daily. This was done so that if at the first counting any were overlooked they would be found and removed at the second.

In the mode tables, the individuals were arranged in groups of 5's or 10's for convenience and to save space. The means used, however, in the calculation of the standard deviation and coefficient of variability were obtained from all the individuals which had been carried out to the second decimal place.

General Life History

The non-sexual or amictic females multiply exclusively by parthenogenesis. Their eggs carry the diploid number of chromosomes, are not capable of fertilization, and produce females, thus multiplication by diploid parthenogenesis is carried on for many generations. However, at times from these eggs another kind of female hatches which produces small eggs that develop into males. These male-producing eggs carry the haploid number of chromosomes. The females which produce them are called mictic and are identical with the amictic females in outward appearance. Their eggs, however, are capable of being fertilized. If the eggs of the mictic females are fertilized, they produce instead of the haploid egg a larger, dark, thick-shelled egg which has the diploid number of chromosomes. This winter egg, as it is called, always develops into a parthenogenetic amictic female.

The entire life of a female lasts usually about seven to eight days depending upon the temperature and other variable external conditions. The first swimming offspring appear from 32 to 57 hours after hatching of the mother. To all outward appearance, the mictic and amictic females of *Hydatina senta* are indistinguishable, but they differ markedly in certain features of their life histories, particularly in the periods of fecundity and the total length of life.

Embryonic Period

The hatching or the embryonic period lasts from the deposition of the egg until hatching. During this period embryonic development is taking place. The length of this period varies from 18 to 26 hours with the mean for 184 amictic females at 22.34 hours, for 113 unfertilized mictic females at 22.53 hours, and for 88 fertilized females at 21.40 hours. This shows that there is in all probability no difference in the hatching time. (See Table I.)

PRE-FECUND PERIOD

The pre-fecund or adolescent period is one of rapid growth. This period extends from the hatching of the egg to the beginning of the fecundity period as shown by the production of the first egg. The length of this period at a temperature of 16° to 17° C. varies from 32 to 57 hours with the mean for 184 amictic females at 41.66 hours, for 110 mictic females at 42.72 hours, for 80 mictic females whose eggs have been fertilized at 46.91 hours. (See Table II.) There was no mortality during this period of immaturity of the 385 individuals studied.

FECUND PERIOD

During the fecund or egg-laying period the eggs are laid one by one. The first were laid from 32 to 57 hours after hatching. After the first egg, additional eggs were produced at varying intervals and this continued for a number of days. The parthenogenetic mictic female deposited 16 to 56 of the small male-producing eggs. The amictic female deposited 16 to 66 of the larger, female-producing eggs. The mictic female whose eggs had been fertilized deposited 3 to 26 of the large, thick-shelled fertilized eggs. The modal fecundity of 184 amictic females is 51 daughters and the mean 45.39 ± .3261. The modal fecundity for 110 mictic females is 46 male young and the mean 42.52 ± .5288. The modal fecundity for 88 mictic females whose eggs have been fertilized is 13 fertilized eggs and the mean 9.98 ± .3015. Thus the mictic and amictic females produce nearly the same mean number of offspring; 42.52 and 45.39 respectively. (See Table IV.) These results are much more nearly in agreement

TABLE I

Comparison of the embryonic or hatching period of amictic, unfertilized mictic, and fertilized mictic females.

Number of hours	17	18	19	20	21	22	23	24	25	26	Total
Amictic females	2	9	1	110	81	57	76	127	10	20	493
Unfertilized mictic females	0	5	1	18	18	7	24	23	3	14	113
Fertilized mictic females	0	4	0	18	33	13	11	5	3	1	88

	Mean	Standard deviation	Coefficient of variability	Mode
Amictic females	22.34±.5105	3.1668±.0381	7.96±.1706	24
Unfertilized mictic females	22.53±.1355	2.13 ±.0957	9.49±.4266	23
Fertilized mictic females	21.40±.1126	1.57 ±.0795	7.33±.3615	21

TABLE II

Comparison of the pre-fecund periods of amictic, unfertilized mictic, and fertilized mictic females

Number of hours	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
Amictic females		1	6	7	4	13	24	3	2	35	11	22	11	5	25	6	3	0	1	0	0	1	0	1	2	1	0
Unfertilized mictic females	4	6	3	2	4	2	0	0	0	17	15	14	10	4	14	4	2	0	1	0	0	0	2	2	1	2	1
Fertilized mictic females	3	0	0	0	0	0	0	0	0	0	1	1	4	4	12	19	8	15	11	0	0	2	0	0	0	0	0

	Mean	Standard deviation	Coefficient of variability	Mode	Total
Amictic females	41.66±.2154	4.333±.1524	10.4±.3642	41	184
Unfertilized mictic females	42.72±.5166	8.047±.3662	18.83±.8579	41	110
Fertilized mictic females	46.91±.2671	3.56 ±.1902	8.29±.4438	47	80

TABLE III

Duration of the period of fecundity of amictic, unfertilized mictic, and fertilized mictic females.

Number of hours	8	13	18	23	28	33	38	43	48	53	58	63	68	73	78	83	88
Amictic females	1	0	0	1	0	0	1	1	5	6	10	12	12	7	29	23	20
Number of hours	93	98	103	108	113	118	123	128	133	138	143	148	153	158	163	Total	
Amictic females	17	11	5	7	3	1	2	3	2	0	1	2	0	1	1	184	

Number of hours	11	16	21	26	31	36	41	46	51	56	61	66	71	76	81	86	91	96	101	106	Total
Unfertilized mictic females	2	0	2	2	0	9	9	5	16	19	15	7	2	8	4	4	2	0	0	1	107

Number of hours	2	7	12	17	22	27	32	37	42	47	52	57	62	67
Fertilized mictic females	1	0	0	0	3	19	0	0	2	4	23	0	0	0
Number of hours	72	77	82	87	92	97	102	107	112	117	122	127	Total	
Fertilized mictic females	9	9	0	0	1	3	5	0	0	0	0	1	79	

	Means	Mode	Standard deviation	Coefficient of variability
Amictic females	81.25 hrs. ±1.125	78 hrs.	22.53 ± .781	27.72 ± .978
Unfertilized mictic females	54.35 hrs. ±1.106	56 hrs.	17.00 ± .785	31.2 ±1.441
Fertilized mictic females	52.87 hrs. ±1.740	52 hrs.	23.02 ±1.232	43.54 ±2.330

than those found by Miller in *Lecane inermis* where the mictic females produced only two-thirds as many offspring as did the amictic females.

The standard deviation for fecundity in 110 mictic females is 8.16 ± .3715 and for the 184 amictic females 6.32 ± .2225. The coefficient of variation for the unfertilized mictic females is 19.22 per cent ± .8761 and for amictic females is 13.92 per cent ± .4931.

The mictic and amictic females mature and deposit the first eggs at about the same time. Since the mictic female produces practically the same number of offspring as the amictic, the fecund period should be the same. The minimum number of hours of the fecund period was 11 for the mictic and 8 for the amictic females. The total number of hours for the mictic ranged from 11 to 106 hours and for the amictic 8 to 163 hours. The mode for the mictics is 56 hours, for the amictics 78 hours. The mean for the fecund period of 107 mictics is 54.35 hours ± 1.106, for the 184 amictics 81.25 hours ± 1.125, and for the 79 mictics whose eggs were fertilized 52.87 hours ± 1.740. The standard deviation is for the amictics 22.53 ± .7819, for the unfertilized mictics 17 ± .7815, and for the mictics whose eggs have been fertilized 23.02 ± 1.232. The coefficient of variation for the amictics is 27.72 ± .9780; for the unfertilized mictics 31.2 ± 1.441; for the mictics whose eggs have been fertilized 43.54 ± 2.330. (See Table III.)

TABLE IV

Comparison of the fecundity of amictic and unfertilized mictic females and the total number of offspring produced during the life time of each of the 184 amictic and 110 unfertilized mictic females. Also showing the number of fertilized eggs produced by each of the 88 fertilized mictic females.

Number of offspring	16	21	26	31	36	41	46	51	56	61	66	Total
Amictic females	1	2	3	0	6	25	59	66	20	1	1	184
Unfertilized mictic females	1	2	5	5	5	14	42	33	3	0	0	110

Number of fertilized eggs	3	8	13	18	23	28	Total
Fertilized mictic females	1	29	47	10	0	1	88

	Mean	Standard deviation	Coefficient of variability	Mode
Amictic females	45.39±.3261	6.32±.2225	13.92±.4931	51
Unfertilized mictic females	42.52±.5288	8.16±.3715	19.22±.8761	46
Fertilized mictic females	9.98±.3015	4.20±.224	42.18±2.138	13

The mictic female requires on the average 26.90 hours less to produce its offspring, although it produces practically the same number

TABLE V

Duration of the post-fecund period of amictic, unfertilized mictic, and fertilized mictic females

Number of hours	5	15	25	35	45	55	65	75	85	95	105	115	125	135	145	155	165	Total
Amictic females	1	11	38	9	22	20	9	13	5	10	10	3	6	1	3	0	2	163

Number of hours	6	16	26	36	46	56	66	76	86	96	106	116	126	136	146	156	166	176	186	196	Total
Unfertilized mictic females	2	5	20	3	10	9	7	10	2	11	6	2	3	1	0	0	0	1	1	1	94

Number of hours	18	28	38	48	58	68	78	88	98	108	118	128	138	148	158	168	178	188	198	Total
Fertilized mictic females	2	25	1	16	2	0	18	1	6	0	0	5	0	1	0	0	0	0	1	78

	Mean	Mode	Standard deviation	Coefficient of variability
Amictic females	53.94 hrs. ± .743	25 hrs.	14.00 ± .524	25.95 ± 1.347
Unfertilized mictic females	59.29 hrs. ± 1.558	26 hrs.	22.40 ± 1.099	37.78 ± 1.719
Fertilized mictic females	56.80 hrs. ± 2.577	28 hrs.	33.63 ± 1.829	59.20 ± 3.020

as the amictic female. This means that the mictic female deposits its small male-producing eggs, on the average, in more rapid succession than the amictic females which produce the larger female-producing eggs. The unfertilized mictic female produces on the average one offspring every 1.2 hours; the amictic female produces on the average one offspring every 1.8 hours; the fertilized egg every 5.2 hours. These figures represent the mean fecundity divided by the mean duration of the fecund period.

Post-fecund Period

The post-fecund period extends from the deposition of the last egg until death of the individual. During the old age period the activities of the female gradually cease, structural degeneration sets in, and death follows usually about the seventh or eighth day. (See Table V.)

In Table V is given the duration of the post-fecund period for all individuals of the amictic and unfertilized mictic females. Seventy-nine amictic females of 184 or 43 per cent died within 36 hours after deposition of the last egg; 37 per cent died within 24 hours, all died within 160 hours. Of the total 163 individuals having a post-fecund period 38 lived 25 hours, which was the mode and the commonest period of death; from 45–55 hours was the next commonest period. The maximum length of the period of old age for the amictics is 160 hours. For the unfertilized mictic it is 192 hours.

The mortality rate reaches one maximal point at about the beginning of the period of old age, perhaps as the result of the exhausting effort of the production of the last eggs. Finally, towards the end of life, it rises to 100 per cent. A large proportion dies immediately after the period of egg-production. But those individuals which pass safely through this period live for some time; in such populations the old females are thick, heavy and sluggish in their movements.

The mictic females cease to deposit eggs earlier, on the average, than the amictic females. Therefore the post-fecund period of the mictic females is extended, being on the average 59.29 hours, while in the amictic it is 53.94 hours.

General Discussion

The amictic and unfertilized mictic females of *Hydatina senta* under controlled conditions differ considerably in length of life, rate of egg production, duration of the fecund and post-fecund period.

In *Hydatina senta*, the difference in the length of life of the amictic and mictic female probably results largely from the differences in the metabolic rates. The amictic females live longer than the mictic females. (See Table VI.)

TABLE VI

Total length of life (from egg to senile adult) of amictic, unfertilized mictic, and fertilized mictic females

Number of hours	87	97	107	117	127	137	147	157	167	177	187	197	207
Number of amictic females	1	0	0	1	4	0	20	16	14	22	8	29	8
Number of hours	217	227	237	247	257	267	277	287	297	307	317	Total	
Number of amictic females	6	14	9	9	4	6	5	5	1	0	2	163	

Number of hours	86	96	106	116	126	136	146	156	166	176	186	
Number of unfertilized mictic females	1	1	1	1	9	2	25	8	12	7	4	
Number of hours	196	206	216	226	236	246	256	266	276	286	296	Total
Number of unfertilized mictic females	11	2	7	9	4	1	1	0	0	0	2	94

Number of hours	77	87	97	107	117	127	137	147	157	167	177	
Number of fertilized mictic females	1	0	2	2	6	1	2	13	0	14	3	
Number of hours	187	197	207	217	227	237	247	257	267	277	287	Total
Number of fertilized mictic females	5	13	0	10	0	3	1	0	3	0	1	78

	Mean	Standard deviation	Coefficient of variability	Mode
Amictic females	192.95±2.194	46.13±.628	23.90 ±.842	197
Unfertilized mictic females	170.53±1.305	19.94±.914	11.106±.509	146
Fertilized mictic females	172.43± .498	6.65±.355	3.85 ±.205	167

In *Lecane inermis*, according to Miller (1931), the difference in the length of life of the amictic and mictic females results largely from the difference in the severity of the process of egg-production, and therefore the mictic females survive the fecund period better. More mictic females live longer because they produce fewer, smaller eggs than the amictic female at a slower rate, and cease egg deposition at an earlier age.

The mictic females of *Hydatina senta* resemble the females of *Lecane inermis* in that some of them live many hours after the cessation of egg production, but the entire length of life of the amictic females covers a longer period than the mictic females, the fecundity period is longer, but the fecundity is practically the same. The relative longevity of the amictic and mictic female of *Hydatina senta* is not correlated with the relative fecundity as in *Lecane inermis*.

Euchlanis triquetra in certain points resembles *Hydatina senta*. According to the observations found by Lehmensick, the amictic and mictic females produce the same number of eggs and live about the same length of time. But the mictic female produces its eggs more rapidly and therefore has a longer post-fecund period. Wesenberg-Lund states (1930) that "if not fertilized, investigations hitherto carried out seem to show that the number of eggs laid by the two sorts of females is almost the same, but that those of the mictic female are laid in a shorter time."

We find that *Hydatina senta* correlates with *Euchlanis triquetra* in that the amictic and mictic females produce practically the same number but that those of the mictic female are laid in a shorter time.

Investigations show that in different species the relative fecundity, length of life, and rate of egg-production of the mictic and amictic female vary greatly. There are probable physiological differences between the two types of females, but as to what the nature of the fundamental differences may be is for further studies to reveal.

SUMMARY

This paper deals with a comparison of the life cycle of the bisexual rotifer, *Hydatina senta*. The three types of individuals, amictic, unfertilized mictic, and fertilized mictic females are compared as to the periods of their life histories.

The mean length of life for the amictic females is 192.95 ± 2.1947 hours; for the unfertilized mictic females 170.43 ± 1.3051 hours; and for the fertilized mictic females 172.43 ± .4983 hours. More than half of the amictic females survive the modal life duration.

The longer life of the amictic females, as compared with the mictic, results from the probable differences in metabolic rate of the

two. The amictic and mictic females produce practically the same number of offspring, the mean fecundity for the amictic is 45.39 ± .3261 and for the unfertilized mictic 42.520 ± .5288.

The mean duration of the fecundity period of the amictic female is 81.25 ± 1.1250 hours with a standard deviation of 22.53 ± .7819 hours and a coefficient of variability of 27.72 per cent. The mean for the unfertilized mictic female is 54.35 ± 1.1061 hours, the standard deviation 17 ± .7851 hours, the coefficient of variability 31.20 per cent.

In summary, the amictic and mictic females produce practically the same number of eggs but those of the mictic female are laid in a shorter time and the amictic female usually lives a longer life. The relative longevity of the amictic and mictic female of *Hydatina senta* is not correlated with the relative fecundity. (See Tables VII and VIII.)

TABLE VII

Comparison of the relation of life duration to fecundity as shown by amictic and unfertilized mictic females.

	Mean length of life	Mean fecundity	Mode length of life	Mode fecundity	Total no. of individuals used
Amictic females.......	192.95 hours	45.385 offspring	197 hours	51 offspring	184
Unfertilized mictic females.............	170.53 hours	42.518 offspring	146 hours	46 offspring	110

TABLE VIII

Comparison of the relation of life duration to rate of offspring production showing the mean time required for the production of an offspring. These figures were obtained by dividing the mean fecundity by the mean duration of the fecund period.

	Mean length of life	Mean rate	Total no. of individuals used
Amictic females............................	192.95 hours	1.8 hours	184
Unfertilized mictic females...................	170.53 hours	1.2 hours	110
Fertilized mictic females.....................	172.43 hours	5.2 hours	88

The mictic females of this species may be fertilized during immaturity. The fecundity of the mictic female is reduced by fertilization; the mean number of eggs being 9.97 ± .3015, standard deviation 4.20 ± .224, and the coefficient of variability 42.18 ± 2.1388. The length of life of the mictic female is not appreciably altered by the production of fertilized eggs. The mean length of life for the fertilized mictic is 172.43 ± .4983 hours and for the unfertilized mictic females 170.53 ± 1.3051 hours.

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THE RATE OF OVIPOSITION IN THE FRUIT FLY, DROSOPHILA

HERBERT SHAPIRO

(*From the Zoölogical Laboratory, Columbia University, and the Marine Biological Laboratory, Woods Hole, Mass.*)

As a subject attracting interest because of economic applications, egg-laying has been studied in the house fly and in other insects with a view to determining the factors affecting the total number of ova deposited. Because of its possible bearing on a question which was formerly under investigation by the author, an examination of the rate of egg production in the fruit fly *Drosophila melanogaster* was undertaken for the determination of individual variations, and the similarities, if any, among different mutants. It is not intended in this account to go into the possible experimental modification of oviposition rate nor to add anything to a treatment of the external influences affecting oviposition in insects in general (Richardson, 1925) but rather to describe the process as found under what may be called "normal" conditions for the life of this genus. Not only because of easy cultivation in the laboratory, eliminating special vivaria as may be required for other insects, but also due to the variety of effective media available (Pearl, 1926; Bridges, 1932) and the small amount of space necessary, *Drosophila* is a form very suitable for the study of egg-laying. Of greater moment is the fact that *Drosophila* lays eggs regularly over a longer period and in larger numbers as compared with the activity of certain other forms in these respects; the Cecropia moth, for example, requires only three or four days to lay its quota of several hundred eggs (Rau, 1910). An interesting and compact account of the biology of the pomace fly and complete references up to 1925 are contained in the monograph of Morgan, Bridges, and Sturtevant (1925); for this reason only a few experiments bearing on this study need be cited.

Castle and associates (1906) demonstrated that close inbreeding did not diminish the fertility of *Drosophila*, provided fertile pairs were selected to continue the stock. For a detailed account of the morphology of the ovary and the internal phenomena of reproduction, reference may be made to the description of Nonidez (1920), and of Laurinat (1931), while the courtship and other external phenomena of reproduction have been observed by Sturtevant (1915, 1921). That factors for egg size exist in all four linkage groups has been brought out by Warren (1924), who also gives a description of the external appearance of the egg. The cytological events accompanying and consequent upon fertilization have been pictured by Huettner (1924).

Before proceeding further, it might be said that the general result of this investigation is that although the life span, active egg-laying period, and total egg output varied greatly from individual to individual in the various crosses, there is found fundamentally the same method of elaborating eggs, which is followed also in the cases of certain invertebrates other than *Drosophila* for which data are available in the literature, and to which reference will again be made. That is, to put the matter another way, a general equation for the rate of laying may be applied to the data secured.

The search for a means of securing the eggs readily and arranged so that they might be counted offered no real difficulty. It was found that if an agar gel was plated out on a small cardboard spoon about three inches long (such as was used, for example, in the experiments of Patterson, 1929) the cardboard would absorb water, leaving crevices in the surface of the gel. It was particularly desirable to avoid this inasmuch as flies will tend to deposit their eggs in these crevices, thereby rendering difficult the procedure of counting. This defect in the technic was remedied by impregnating the spoons with paraffin of high melting point (68°–70° C.). The medium upon which the flies could live and lay their eggs was prepared by adding 5 per cent of molasses to a hot 1 per cent solution of agar in distilled water. About 175 cc. of this medium would suffice for 110 spoons, into which the medium was poured and allowed to gel, forming a plate which adhered to the spoon, but which might, if desired, be cleanly and easily separated from the spoon in which it was cast. After cooling, a drop of yeast suspension in distilled water was placed on the surface of the "plate," which was tilted to allow the suspension to spread uniformly over the surface. These spoons, made up thus each evening, were then placed in crystallizing dishes, carefully covered to prevent access of foreign flies, and used the following day. The flies were secured soon after hatching, and placed individually in a vial four inches long and an inch in diameter together with one of the spoons. The vial was then plugged with cotton. At approximately 24-hour intervals throughout the life of the fly, the spoon upon the surface of which the eggs were deposited was removed from the vial and a fresh spoon introduced. The eggs could then easily be counted under a binocular. Table I indicates how counts were recorded and includes also some derived data. Frequent examinations in the early stages of the work revealed no deposition of eggs on the walls of the vial or

TABLE I

A sample record. From the data (of an individual of cross *k*) presented in this table, the curves in Figs. 1 to 3 were drawn.

Date (1930)	Hour of removal of eggs	Number of eggs counted	Time elapsed	Total No. of eggs	$\frac{\text{Time}}{\ln \text{total}}$
			days		
7/16	3:50 P.M.	11	1.00	11	0.4170
7/17	1:58 P.M.	70	1.92	81	0.4369
7/18	2:34 P.M.	102	2.95	183	0.5663
7/19	3:05 P.M.	125	3.98	308	0.6946
7/20	3:02 P.M.	116	4.98	424	0.8232
7/21	2:41 P.M.	148	5.97	572	0.9403
7/22	2:06 P.M.	102	6.96	674	1.069
7/23	2:15 P.M.	136	7.97	810	1.190
7/24	2:17 P.M.	115	8.97	925	1.313
7/25	2:47 P.M.	126	10.00	1051	1.437
7/26	3:06 P.M.	127	11.01	1178	1.557
7/27	3:03 P.M.	124	12.01	1302	1.675
7/28	12:28 P.M.	78	12.90	1380	1.784
7/29	2:51 P.M.	139	14.00	1519	1.911
7/30	2:57 P.M.	92	15.01	1611	2.032
7/31	10:18 A.M.	72	15.81	1683	2.129
8/1	12:03 P.M.	93	16.88	1776	2.256
8/2	10:42 A.M.	98	17.82	1874	2.365
8/3	12:08 P.M.	87	18.88	1961	2.491
8/4	11:38 A.M.	83	19.86	2044	2.606
8/5	11:27 A.M.	61	20.85	2105	2.725
8/6	11:09 A.M.	79	21.84	2184	2.840
8/7	11:25 A.M.	46	22.85	2230	2.964
8/8	11:30 A.M.	57	23.85	2287	3.083
8/9	10:08 A.M.	51	24.79	2339	3.196
8/10	11:11 A.M.	42	25.84	2380	3.324
8/11	12:10 P.M.	30	26.88	2410	3.452
8/12	11:43 A.M.	44	27.86	2454	3.569
8/13	10:46 A.M.	29	28.82	2483	3.687
8/14	11:07 A.M.	41	29.84	2524	3.809
8/15	11:09 A.M.	35	30.84	2559	3.930
8/16	9:41 A.M.	16	31.78	2575	4.047
8/17	11:07 A.M.	25	32.84	2600	4.176
8/18	10:43 A.M.	16	33.82	2616	4.298
8/19	10:50 A.M.	14	34.82	2630	4.421
8/20	10:40 A.M.	11	35.81	2641	4.545
8/21	11:27 A.M.	8	36.84	2649	4.675
8/22	11:04 A.M.	6	37.83	2655	4.798
8/23	11:08 A.M.	7	38.84	2662	4.925
8/24	10:01 A.M.	4	39.79	2666	5.044
8/25	10:43 A.M.	7	40.82	2673	5.173
8/26	10:47 A.M.	2	41.82	2675	5.299
8/27	10:58 A.M.	1	42.83	2676	5.427
8/28	10:20 A.M.	6	43.80	2682	5.548
8/29	11:29 A.M.	2	44.85	2684	5.681
8/30	10:00 A.M.	0	45.78	2684	5.799
8/31	10:28 A.M.	0	46.81	2684	5.929
9/1	11:07 A.M.	0	47.84	2684	6.059
9/2	11:46 A.M.	dead	48.88	2684	6.191

elsewhere on the spoon other than the surface of the gel, so that search for eggs in these locations was not thereafter regularly made. The eggs, being white, and 0.5 mm. in length, stand out clearly against the brown medium. Occasionally recounts were made, and it was found that quite consistently all the eggs would be included in a count. In handling the vials there was a minimum of shaking to avoid any possibility of disturbing the laying activities of the flies. The work was greatly facilitated by the use of an incubator in which the flies were kept at 24° C. ± 1° C.; and for preventing the vials from rolling about, and to maintain the surface of the medium level, by the availability of racks which had been designed by Doctor Calvin B. Bridges, and kindly loaned by him.

The results reported here comprise observations on the complete egg-laying history of some 93 females. Virgins, mated flies, and flies kept with more than one male were followed; while in the case of *Drosophila obscura*, which has been studied extensively by Lancefield (1929), both species or physiological races, Race *A* and Race *B*, were observed. Thus with *Drosophila melanogaster*, these crosses were made:

a. Type (wild) females (virgins).
b. + ♂ × + ♀ (♂ removed after mating).
c. + ♂ × + ♀ (with male).
d. + ♂ × + ♀ (with 2 males. In all cases males were replaced if their death occurred before that of the female.)
e. Vestigial females (virgins).
f. vg ♂ × vg ♀ (with male).
g. + ♂ × vg ♀ (with male).
h. + ♀ × vg ♂ (with male).
i. Sepia ♂ × sepia ♀ (with male).
j. Siblings resulting from cross *h* were mated to each other (with male).
k. Siblings resulting from cross *g* were mated to each other (with male).
l. Lobe^2 ♂ × Lobe^2 ♀ (with male).

With *D. obscura:*

a. Race *A* ♂ × Race *A* ♀ (with ♂).
b. Race *B* ♂ × Race *B* ♀ (with ♂).
c. Race *A* ♂ × Race *B* ♀ (with ♂).
d. Race *B* ♂ × Race *A* ♀ (with ♂).

A few details of passing interest may be dealt with briefly. After the death of most of the flies the abdomen was opened, the ovaries dissected out, and the number of remaining well-formed eggs counted.

No correlation between this variable and any of the other factors studied could be found. The females varied with respect to their actual egg-laying period, some ceasing to produce eggs several or more days before death, others still laying, though at a much diminished rate, even to the end of the life span. Virgins, as well as mated flies, lay eggs regularly, a fact noted previously both by Hyde (1921) and by Hanson and Ferris (1929). The former remarks, "The fecundity record is apparently not modified as a result of fertilization for the unfertilized female lays eggs regularly and in large numbers," possibly implying that mating has no effect on the rate of laying, a point which will be dealt with again. Complete sterility was found in three flies; two of these contained mature eggs in the abdomen after death. In the third, which had large brown concretions around the spermathecæ, the ventral receptacle and spermathecæ were packed with motionless sperm.

The hybrid females (crosses *j* and *k*) displayed their vigor not only in the increased egg production, which was of the order of two or three times that of the mother or of the females of the stock from which the male parent was selected, but also by their incessant activity in the vials in which they were kept. It was more difficult to replace spoons from these vials than from others, an active fly being unfortunately, quicker than the hand.

In *D. melanogaster* laying commenced in general one to two days after hatching, though this period is prolonged in the case of some of the virgins to seven or eight days. Race *B* of *D. obscura* usually precedes egg-laying by a characteristic period of three days of non-productivity, while Race *A* females wait two days before giving off the first egg.

Figure 1 represents the egg-laying of one of the hybrids of cross *k*—a total of 2,684 eggs in a life of about 49 days. The set of data given by this fly was used also in plotting Figs. 2 and 3. In the first figure, where the total number of eggs is plotted against time, a sigmoid curve results. A similar figure appears for all the other flies, and all the data might indeed be represented in this fashion as a family of curves. For this purpose, however, a more convenient means of plotting is utilized, as will be seen in the last figure. A phenomenon peculiar to *D. obscura* appears also in the curves plotted for them. Most of the females of this genus are in the habit of stopping egg-laying at irregular intervals for a period of one or two days, and very occasionally, longer. This, nevertheless, does not affect the characteristic shape of the curves. These curves, as well as those succeeding, are quite generally representative of the entire group of curves drawn.

One would like to know the appearance of the daily egg-laying curve. Inasmuch as it was not practicable to make measurements at exact 24-hour intervals, and also because a further and more serious objection offers itself in the tendency of flies to lay eggs in irregular spurts, as described by Adolph (1920), who says, "There is a distinct tendency for an individual fly to lay several eggs within a few minutes.

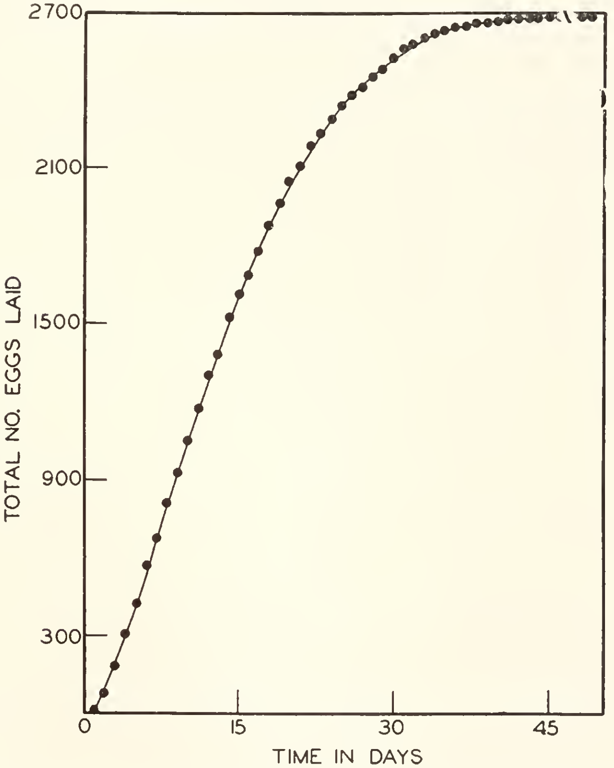

FIG. 1. The total number of eggs produced during the lifetime of a fly. These sigmoid integral curves are obtained also when the data from other individuals are similarly plotted. Figs. 1 to 3 inclusive are curves drawn from the records (contained in Table I) for the same female.

How often such spasms occur under uniformly stimulating conditions is unknown, but there is certainly no daily or weekly periodicity among different individuals," the data were not plotted simply as recorded. Guyénot (1913) remarks, ". . . la ponte se produit sous forme de décharges, causées par la surabondance des oeufs formés." The following means, consequently, were adopted for obtaining the curve shown in Fig. 2, a procedure which may be justified by a simple hypothetical consideration, inadequate as it may be. The tendency to lay eggs in bunches has been noted. Thus, if at the height of its egg-laying period, a fly laid 120 eggs per day, it would not lay one egg regularly every twelve minutes as might be computed, but rather several eggs during this interval, and then none for perhaps the next half hour. For this reason one must have recourse to an integral curve, for considered from the standpoint of minutes, the egg-laying is irregular, but from the standpoint of days or weeks, it becomes quite regular.

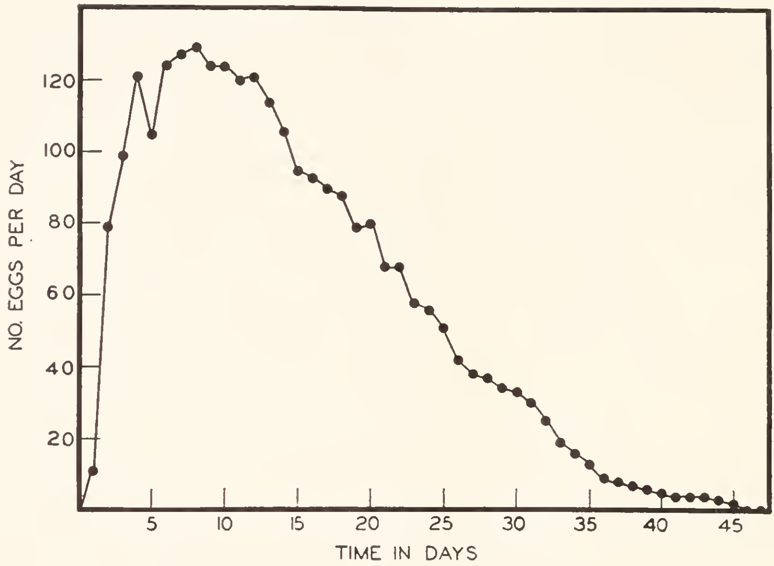

FIG. 2. The daily egg production. This curve was secured by graphical interpolation from an enlarged plot of Fig. 1.

A large integral curve similar to that shown in Fig. 1 was plotted on paper of dimensions $3\frac{1}{2} \times 4$ feet, and then, by use of the mirror tangentimeter described by Latshaw (1925), the number of eggs for each 24-hour interval could be pieced out of the curve with fair precision. The differential curve so secured rises to a maximum of about 130 eggs per day and then falls off gradually to zero at about the end of the fly's life. The area under the curve would represent the total number of eggs deposited. In the attempt to arrive at some general conclusions from the data, the figures were set up in a manner of which Table II is an example. No clean-cut generalizations suggested themselves from a study of such averages. With the apparent general similarity of the type of curve shown in Fig. 1 as drawn for different individuals in mind, it was decided to study individual cases, to determine whether some general relationship might be found into which they all would fit.

It is found that if $t/\ln T$ is plotted against t, where t represents the time in days at which any given total (T) is attained, the points arrange themselves linearly, as may be seen by inspection of Fig. 3. It will be noted that the last four points are off the curve. This is due to the cessation, on the forty-fifth day, of egg-laying by the fly, which lived four days longer; the point for each of these post-laying days was calculated by using the same total, and these points arrange themselves along another line. The curve shown in Fig. 3 has been drawn also for each of the other 92 females studied and gives an equally good fit for these too, except three others where the points scatter rather more widely on each side of the line. All the sets of data might be plotted in somewhat fan-shaped arrangement in the space of Fig. 3, those representing a smaller rate lying above the curve there drawn, those with a greater rate falling below. If instead of this the slope of each curve so obtained be plotted against the grand total of eggs produced, the points fall as found in Fig. 4.

TABLE II

Cross *c* : + ♂ × + ♀ (with male)

Total number of eggs laid	Life span	Day of cessation of egg-laying
	days	
755	27	27
617	20	18
1176	41	28
1537	41	40
830	36	33
1097	35	32
Average 1002±109(A.D.)	35±2.5(A.D.)	29.7±1.2(A.D.)

From Fig. 3 it follows that

$$\frac{t}{\ln T} = at + b,$$

where t and T remain with the same meaning indicated previously, a and b representing respectively the slope of the curve and its intercept on the y axis. This yields an equation for the total number of eggs (T) already produced at any stated time (t):

$$T = e^{\frac{t}{at+b}}.$$

To get the change in total with time, the first derivative is taken

$$\frac{dT}{dt} = \frac{be^{\overline{at+b}}}{(at+b)^2},$$

(the overline carries t above it: exponent $\frac{t}{at+b}$)

and to arrive at the time at which the rate of egg-laying is at its maximum, the second derivative is taken, set equal to zero, and solved

$$\frac{d^2T}{dt^2} = \frac{be^{\frac{t}{at+b}}[b - 2a(at+b)]}{(at+b)^4},$$

$$t = \frac{b}{2a^2}(1 - 2a)$$

For the fly whose data are plotted as shown in the figures, this maximum is thus calculated to be 5.7 days, when the values used for the constants *a* and *b* are 0.121 and 0.221, whereas the value found by inspection of Fig. 2 is about 7.5 days.

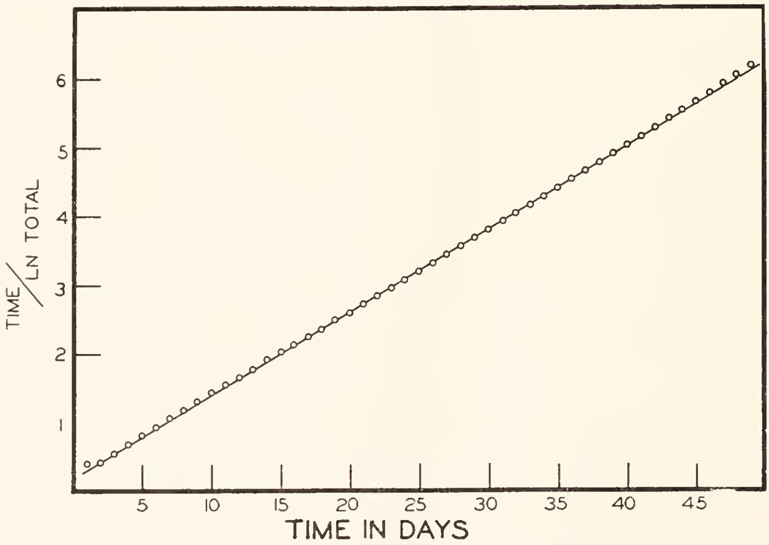

FIG. 3. A typical curve, demonstrating the linear relationship between the variables indicated.

The oviposition data are tabulated in the literature for the cases of some other invertebrates. These have been examined and found to fit the formulation just shown. They are as follows:

Hyde (1921) reported what were considered three unusual cases of fecundity for mated *D. melanogaster* females, namely, totals of 1,613, 1,807, and 2,184 eggs. Similar values have been found quite regularly in the animals studied here, and with respect to the hybrids it is rather the rule for them to lay 2,000 to 3,400 eggs. Fauré-Fremiet and Garrault (1928) give data for the egg production of *Margaropus australis*, an acarid. While studying the growth of the snail *Lymnæa columella*, Baily (1931) recorded also the egg-laying. The empirical equation applies also to the data presented by these authors, though not quite so well for some of the individuals of the last-mentioned instance as for *Drosophila* due to the more pronouncedly intermittent character of egg production in the snails; however, there is a definite fit. A curve plotted for one of the sets of data presented by each of these authors is presented in Fig. 5, where it may be compared, for example, with the curve for the data derived from a female of cross *a* (*D. obscura*).

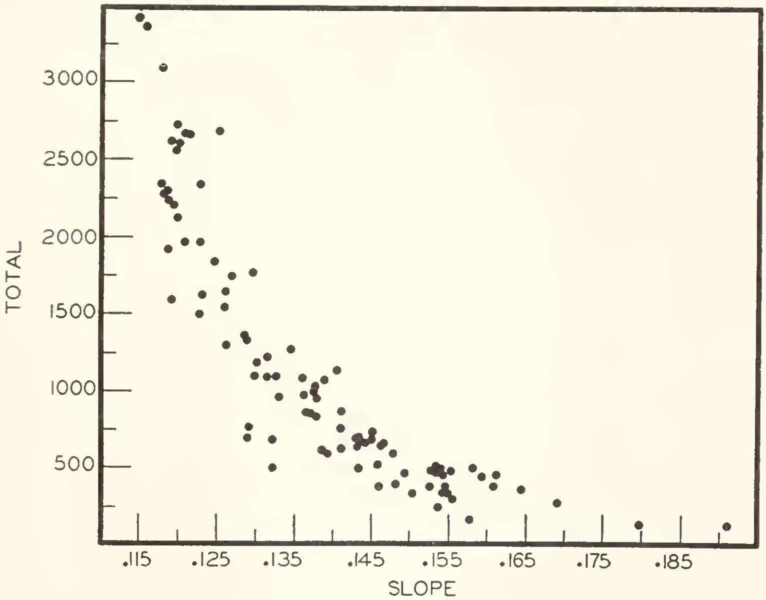

FIG. 4. Each point represents the slope (of a curve plotted as in Fig. 3) in relation to the sum total of eggs produced, for each of the flies studied.

It is to be pointed out here that it follows from what has been described that unless one is certain that females are producing eggs at the same rate, it is not a sound procedure to select flies at random from a stock and use some for experimentation dealing with egg production, and others for controls, and then to compare the averages of the results. This comparison might possibly lead to erroneous conclusions. From the nature of Fig. 2, it becomes evident that it should be determined that the shapes of the egg-laying curves for both controls and experimental material, both as regards height of the maximum and length of the curve, are reasonably similar at the outset. In connection with this, an analogous situation may be cited. Davenport (1931) inveighs against the procedure of drawing conclusions concerning growth processes from accumulated data. From the mass statistics of 100,000 children, one might decide that the velocity of growth is greatest at two periods, one in intra-uterine life, and one at about 14.5 years (in the case of the male). When, however, the study

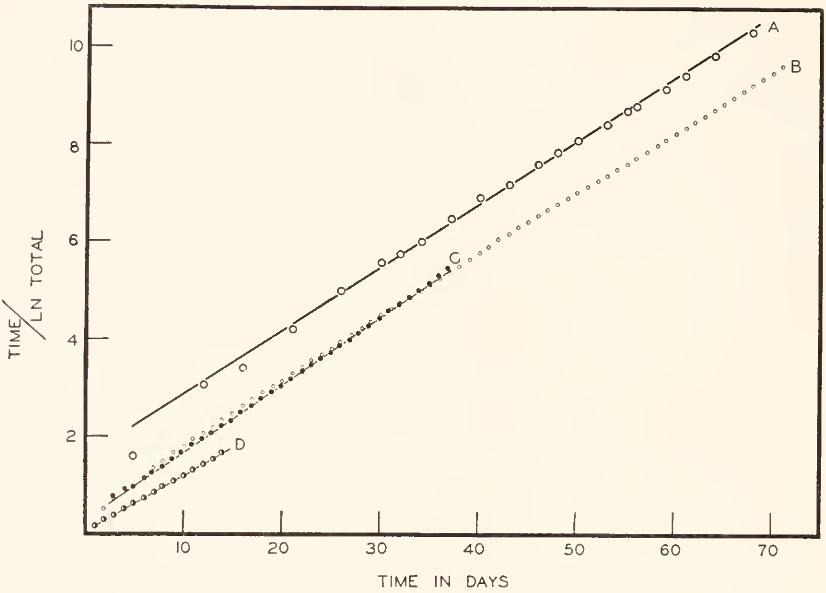

FIG. 5. Comparison of the curves of various invertebrates. *A*, of a snail *Lymnæa columella* (data of Baily); *B*, of *D. melanogaster* (data of Hyde); *C*, of *D, obscura* (Race A); *D*, of a tick, *Margaropus australis* (data of Fauré-Fremiet and Garrault). In *A* the egg output amounted to 725; in *D*, 4346 eggs were produced.

of individual children is made, the resulting curve of growth is found to be very different from that of the mass curves, and varies with different children. Instead of the maximum being reached rather gradually at the age of 14.5 years, a rapid growth of the individual at adolescence, of almost explosive rapidity, is found; the age at which this occurs, and its magnitude, varying with the individual.

When one is dealing with a constantly changing quantity, and where this rate of change will vary for different flies at the same age, the averaging of data will give only a very approximate idea of what is occurring in general in individual cases. If the proper flies be used, one may see that by averaging different kinds of curves such as are presented in Fig. 2, provided their maxima and duration be different, any kind of average curve, within limits, may be produced. This may perhaps account for the differences observed by Hanson and Ferris (1929), when in one experiment the averaged laying curve for mated white flies rose to a maximum of about 24 eggs per day, while in another similar experiment a maximum of about 52 eggs per day was reached. The maxima of averaged curves may be shifted depending on the nature of the maxima of the individual curves being averaged.

Hanson and Ferris decide from their own data presented as averages that mating results in heightened productivity by the female. There is lack of agreement as to whether or not, in general, the male serves to stimulate an increased production of eggs. Guyénot (1913) states that there is a considerable delay, which varies from case to case, in the initiation of egg-laying by certain *Drosophila* virgins, but that, once commenced, their production gradually approaches in intensity that of the mated individual. In the cases observed by the writer, the vestigial virgins started to lay eggs the second day after hatching, and produced eggs regularly thereafter, whereas type virgins delayed laying from two to eight days after hatching. It is of interest to note that examination of the data shows that certain of the virgins produced more eggs during their life than did mated flies, and this in females from a stock which had been well inbred; although the averages were greater for mated flies than for virgins. These figures, with the deviation of the mean (A.D.), are listed here; the numbers in parentheses represent the number of individuals whose data are averaged.

+ virgins	677 ± 116	(8).
+ ♂ × + ♀ (without male)	718 ± 103	(12).
+ ♂ × + ♀ (with male)	1091 ± 115	(7).
+ ♂ × + ♀ (with two males)	1402 ± 98	(4).

Inasmuch as certain virgins, for example, will lay many more eggs than certain mated flies, one would hesitate to reach the generalization from these averages alone that mating stimulates the female to increased egg production, and cannot help suspecting that the increase may not be an intrinsic one. Eight type virgins laid the following numbers of eggs in the time in days indicated in parentheses: 165 (47), 378 (28), 400 (55), 454 (70), 698 (40), 758 (42), 1088 (70), 1474 (31). In view of the great individual variations, the averaging of the records of much greater numbers of individuals would be required to give a definite statistical answer. It might seem that a means may be afforded for obtaining an indication of the course of events, in *Drosophila* at least, by use of the equation described above. If mating results in increasing constantly and continuously the rate of egg production, then plotting as in Fig. 3 the data of an experiment where a virgin lays eggs for a given period and is then mated, there should be a break in the line toward the abscissa after the time of mating. Due, however, to the logarithmic nature of the plot, small deviations or changes in the rate do not become readily manifest; consequently this bend is not accentuated sufficiently to permit a definite and unequivocal separation of the parts of the curve that would result.

In an investigation of egg-laying in the domestic fowl, Fauré-Fremiet and Kaufman (1928) advance the interesting hypothesis supported by histological evidence of what is termed a constant probability of transformation of oöcytes, an interpretation entirely independent of the idea of senescence as offered by Brody, Henderson, and Kempster (1923). In seeking a factual basis for this idea, the first-mentioned authors studied the formation of oöcytes in the hen, and the initial number of oöcytes, and proposed an interpretation of the curve of laying according to such data. After a review of the experimental work done on this phase of the subject, it appears that the activity of the germinal epithelium can be restored in certain pathological or experimental conditions (Pearl, 1921), but they conclude that in the hen, under normal conditions, the number of oöcytes is quite limited after birth. Subsequent to birth, oöcytes of the chick grow slowly and progressively. During the period of egg-laying (which may be of eight years' duration) some of the oöcytes undergo a very rapid growth and increase their vitelline mass a hundred-fold in five to eight days, following which they may be laid.

They propose then the following equation, similar to that of Brody and collaborators, to describe the decrease in rate of egg-laying:

$$N_t = N_o e^{-Kt},$$

where N_t = the number of oöcytes still available at time t (reckoned in years), N_o = the initial number of available oöcytes, e = the base of natural logarithms, and K = a constant. The average curve of decrease in a given race in an individual is said then to depend on two values: N_o, the initial number, representing the stock of available oöcytes; the other they term the probability of transformation of oöcytes, a meaning which is attached to the value K, and $1/K$ or θ, would represent the average life of the oöcyte, or the average period during which the oöcytes can remain at the initial state before undergoing the very rapid growth of yolk accumulation. These constants are supposedly independent of environment, and are probably, according to the authors, hereditary. It is apparent that this equation is formally tantamount to that for a first order reaction, *viz.*,

$$K = \frac{1}{t} \ln \frac{a}{a - x},$$

where the rate of change of the concentration of substance A at any instant is proportional to its concentration at that instant; t represents time, a the original molar concentration, and $(a - x)$ the concentration of A after t minutes. When stated in terms of egg-laying this would mean simply, aside from any implications of a chemically analogous factual basis, that at any instant the rate of decrease of the number of eggs laid is proportional to the number of eggs remaining unlaid.

This equation proposed by Brody, Henderson and Kempster, or the equivalent one of Fauré-Fremiet and Kaufman, for averaged data of egg production by the hen, cannot be carried over to the situation presenting itself in *Drosophila*, where the rise in egg-laying to a maximum is a regular, intrinsic part of the process. The equation of Fauré-Fremiet and Kaufman can be applied only to the descending portion of the curve of laying of the tick *Margaropus australis*, whereas the equation for *Drosophila* can apply also to the entire curve for *Margaropus*. In the case of the arthropod, then, the hypothesis of a probability of transformation of oöcytes appears to be inadequate. To bridge the discrepancy between the curve for laying of *Margaropus* and of the hen, Fauré-Fremiet and Garrault (1928) introduce the conception of the progressive development of a "physiological factor," that is to say, of a complex of somatic conditions allowing yolk accumulation, as being probably responsible for the ascending portion of the curve. Inasmuch as a general equation is found which applies to the whole process, it is perhaps more desirable to conceive it as continuous, and operating throughout as part of the same mechanism, rather than to introduce the idea of a dichotomy, the operation of the second process remaining in abeyance until the completion of the first. However, the existence of such a factor as the first, not yet yielding to exact treatment, is by no means excluded.

Grateful acknowledgment is due Professor D. E. Lancefield and Professor A. H. Sturtevant for reading and criticizing the manuscript.

SUMMARY

The rates of egg-laying of certain mutants of *Drosophila melanogaster* and of two races of *Drosophila obscura* were studied and compared with certain other cases for which data are presented by the authors.

The fecundity of hybrids of *Drosophila melanogaster* was also studied. An empirical equation describes the egg-laying curves of all the flies studied (about 93 in number) and is $T = e^{\overline{at+b}}$ where T represents the total number of eggs already laid at the time t, and e is the base of natural logarithms. The constants a are shown to be correlated with the total number of eggs deposited.

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A NOTE ON THE THYROID GLAND OF THE SWORDFISH (XIPHIAS GLADIUS, L.)

WILLIAM H. F. ADDISON AND MAURICE N. RICHTER

(*From the Marine Biological Laboratory, Woods Hole, the Department of Anatomy, University of Pennsylvania, and the Department of Pathology, Columbia University*)

In teleosts, the thyroid gland is not so well defined as in elasmobranchs and mammals. As a rule the follicles, in smaller or larger groups, are distributed within an abundance of soft connective tissue, and the boundaries of the thyroid tissue are formed by adjacent structures, not by a distinct capsule. McKenzie (1884) in the siluroid, *Ameiurus*, describes the frame-work as consisting of loose connective tissue which does not form a limiting membrane, but merely passes over into the tissue surrounding the adjacent parts. The thyroid vesicles are scattered through this tissue, showing a tendency to arrange themselves in short rows. Gudernatsch (1911) made an extensive study of the distribution of the thyroid tissue in twenty-nine species of teleosts, belonging to twenty families, and found great variation in the compactness of the tissues, ranging from a complete dispersion of the follicles to a rather compact union of them. Compared with the fishes studied by Gudernatsch, the swordfish, *Xiphias gladius*, is noteworthy among the teleosts because of the concentrated character of the gland and its large size.

In dissecting the heads of swordfish at the Marine Biological Laboratory at Woods Hole, we noticed that the main mass of the thyroid gland formed a large fairly well-circumscribed mass of tissue. It was situated in close relation to the cephalic end of the ventral aorta, and partially encircled it. The color was dark red, due to its great vascularity. Its consistency was moderately soft, but with care it could be separated from the surrounding structures. For convenience in dissecting, it was usually left attached to the ventral aorta.

The general appearance in cross-section and the relation of the thyroid to the ventral aorta are shown in Fig. 1. This specimen was from a medium-sized swordfish weighing 330 pounds after the head, tail, and viscera had been removed. After preservation in 10 per cent formalin the thyroid was a firm mass of a dark gray color. It measured 40 mm. in the sagittal direction and 35 mm. in its widest transverse part. The greatest thickness of the thyroid tissue was 17 mm. The organ was composed, for the most part, of four fairly well-defined

PLATE I

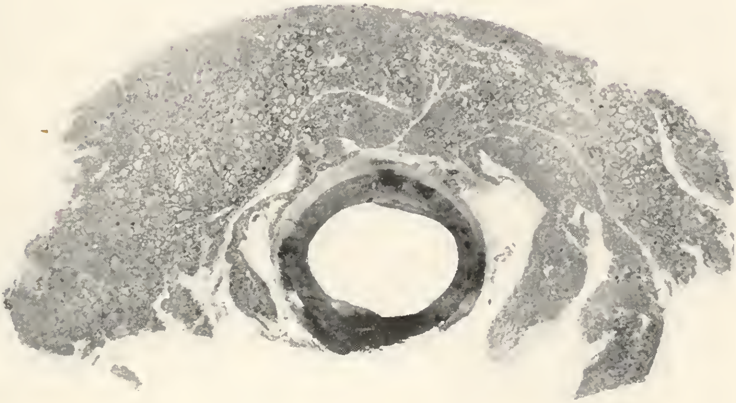

FIG. 1. Cross-section of the thyroid gland of the swordfish partly surrounding the cephalic end of the ventral aorta. × 4.

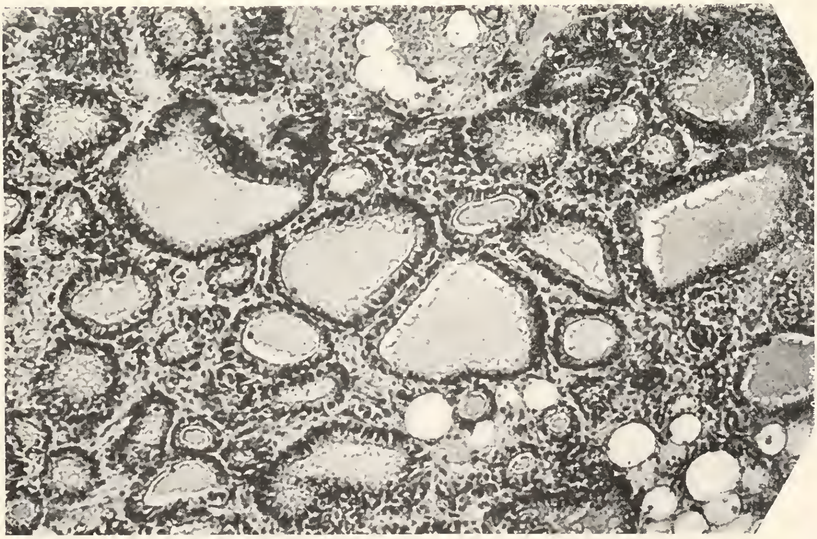

FIG. 2. A small portion of the thyroid gland of the swordfish, showing the compact arrangement of the follicles. × 200.

masses, which were separated from each other by thin connective tissue septa, continuous with the peripheral connective tissue. The two anterior masses, situated on either side of the median line, between the first and second branchial vessels, were larger than the two posterior masses. The latter extended between the second branchial arteries and the single stem for the third and fourth branchial vessels. Each of the four masses consisted of smaller closely-adherent masses or lobules, which were composed of compact thyroid tissue.

Microscopically, the main gland mass shows epithelial-lined follicles containing colloid material (Fig. 2). Around the follicles is a very vascular but scanty supporting tissue in which are a few fat cells. Thus, in certain features, the gland resembles closely elasmobranch and mammalian thyroids. In transverse sections across the entire mass, separate small groups and rows of follicles were also seen. These were scattered in the loose connective tissue between the main part of the organ and the wall of the aorta and the branchial vessel, and showed the typical teleostean arrangement.

The lumina of the follicles vary greatly in size. Of those containing colloid, a small one may measure 30 x 25 μ, a large one 300 x 200μ. The largest ones may measure several millimeters in length. One was found to be 3.5 x 1 mm., and another 2.5 x 0.75 mm. A great number, however, are of intermediate size, measuring about 120 x 80 μ. The general shape is round or oval, the large ones being usually elongated. The walls of the follicles are formed of simple columnar epithelium, which apparently does not rest on a basement membrane.

The epithelial cells are columnar in form, and average 15 x 4.5 μ after fixation in formol-Zenker. The cells are taller and narrower than the "chief" cells of *Mustelus*. According to Ferguson (1911) the latter measure 6–10 μ in height; after fixation in formol-Zenker, however, we find the *Mustelus* cells to be somewhat higher, 13–14 μ. They present many slight variations in shape, some being fusiform, others slightly curved. At places there is a pseudo-stratified appearance, because the nuclei of adjoining cells are alternately higher and lower in position in the cells. The cytoplasm, after staining with Dominici's stain or eosin-azur, is basophilic in the basal portion, and acidophilic towards the lumen. Sometimes the acidophilic zone is seen at the basal margin of the cells. This suggests a reversal of polarity in these cells. In a few cells, the portion of the cytoplasm adjoining the lumen contains acidophile substance in the form of globular masses of varying sizes. These globular masses resemble colloid in appearance. In Gudernatsch's study (1911), the "colloid" cells of Hurthle or of Langendorff were seldom seen. Typical ones are not seen here. The nuclei are usually situated in the center or in the basal third of the cell-body, and contain relatively little chromophilic substance.

In the follicles one usually finds homogeneous retracted colloid material. Within the colloid material in some, there are lighter staining spherical areas or vacuoles. Frequently, groups of ill-defined epithelial cells are also seen within the colloid, as well as hemorrhagic masses of blood cells. Such follicles often lie deeply in the lobule where they would be well protected from mechanical injury.

Throughout the greater part of the gland, the epithelium is separated from the blood by only the endothelium of the thin-walled vessels. The blood-vessels, however, are numerous, and are of relatively large size. Lymph-vessels containing colloid are also seen. The amount of fibrous tissue between the follicles is very small. In addition to the interfollicular supporting tissue there are wider strands of connective tissue between the lobules. In these interlobular strands are sometimes rows of fat cells and single follicles, or small groups of them. Around the peripheral lobules, the connective tissue fibers are arranged in a parallel manner, somewhat closer together than in the adjoining loose connective tissue. After orcein staining, elastic fibers of small size are demonstrable. These are much thinner than those in the walls of the blood-vessels, and are arranged in a loose network. This peripheral connective tissue could scarcely be regarded as a true capsule, but it is somewhat modified from the ordinary loose connective tissue. The consistency of the organ is thus due more to the close arrangement of the follicles than to the presence of a definite peripheral covering membrane.

Pigment cells are distributed in the loose connective tissue around the organ, and are often perivascular in position, in some places lying against the walls of the blood-vessels.

Studies in the thyroid of fishes have revealed several interesting conditions. In the thyroid of the dogfish, A. T. Cameron (1913) finds by chemical analysis that the iodine content is higher than in that of any mammalian thyroid yet examined. Marine and Lenhart (1911) find that hyperplasia of the thyroid tissue develops in brook trout (*Salvelinus*) kept under certain conditions, and that iodine stops the hyperplasia and causes the thyroid to return to the colloid or resting state. For chemical or physiological studies of the teleostean thyroid it would appear that the swordfish thyroid, on account of its large size and the homogeneous structure of the main part of the organ, would afford satisfactory material.

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INFLUENCE OF HYPOPHYSECTOMY ON THE PANCREATIC DIABETES OF DOGFISH

OSCAR ORIAS[1]

(*From the Marine Biological Laboratory, Woods Hole, and the Laboratories of Physiology in the Harvard Medical School*)

Using toads and dogs, Houssay and Biasotti (1930*a*, 1930*b*) have shown the marked influence of hypophysectomy on the course of the diabetes produced by pancreatectomy. In their pancreatectomized animals the diabetes was definitely milder when the hypophysis had been removed. It was the purpose of this investigation to determine whether or not the general conclusions reached by these investigators were applicable to a lower form. The smooth dogfish (*Mustelus canis*) was chosen on account of its availability and its position in the vertebrate scale. Furthermore, its cartilaginous skull renders hypophysectomy a comparatively simple procedure.

Pancreas, Hypophysis, and Glycemia in Elasmobranch Fishes

Elasmobranchs have a large pancreas, its tissue being made up of glandular acini and of insular cells functionally equivalent to the islets of Langerhans in the pancreas of mammals (Jackson, 1922). The isolation and removal of the entire pancreas are procedures easily performed without any serious bleeding. Herring (1911) has shown that the hypophysis of elasmobranchs is developed almost entirely from Rathke's pouch. This same investigator (Herring, 1913) states that "the elasmobranch pituitary differs from all other pituitaries in not possessing a posterior lobe. The brain wall of the embryo merely evaginates to form a paired saccus vasculosus, but no pars nervosa is formed." And although de Beer (1926) showed that there is an extension of neuroglia fibers from the border of the infundibular cavity which penetrates the posterior lobe (pars intermedia), it is certainly true that the selachian lacks a true pars nervosa. This means that one has to deal in this form with a hypophysis composed of (*a*) an anterior lobe of eosinophile and basophile cells, (*b*) a posterior lobe (pars intermedia) of basophile cells, and (*c*) a ventral lobe composed also of basophile cells, but containing, in addition, certain curious large cells staining with eosin and of undetermined significance (de Beer, 1926). In the hypophysectomies of the present investigation no attempt was made to discriminate between these various parts. All removals of the gland have been total, and were performed according to the technique described by Lundstrom and Bard (1932).

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[1] Fellow of the Rockefeller Foundation from Argentina.

The normal glycemia in fish has not yet been satisfactorily worked out. All the recent studies dealing with the blood sugar of fishes have shown great discrepancies between the values found not only among specimens of different species, but also among different individuals belonging to the same species. Although no one has been able to determine precisely all the factors responsible for these discrepancies, undoubtedly the variable degrees of asphyxiation involved in the process of obtaining the blood samples for analysis stand as a cause of paramount importance in explaining the widespread variability of results. There is no doubt that asphyxia induces a condition of hyperglycemia which may last during a period of several days even when the fish is replaced in sufficiently oxygenated water. This has been shown by McCormick and Macleod (1925), Simpson (1926), and Kisch (1929). The asphyxial blood-sugar rise is due to a mobilization of glycogen from the liver (Simpson, 1928; Kisch, 1929).

Apart from the influence of asphyxia, the blood-sugar level seems to vary with the different species of fish because of the differences in their habits of life. According to the investigations of Gray and Hall (1930), who studied fifteen species of teleosts, fast-swimming fishes depending on the speed of their movements to catch their prey have a higher blood-sugar level than those less active bottom-feeders that live on crustaceans and other slow-moving creatures.

Before proceeding to the main problem, an attempt was made to determine the normal blood-sugar level of *Mustelus canis*. Blood samples were directly withdrawn from the heart by means of a syringe with the needle inserted through the ventral median line at the anterior edge of the pectoral girdle. As a rule 0.5 cc. of blood was used for each determination. The reducing substances of blood after precipitation of proteins were determined by the Shaffer and Hartmann method (1921) and computed as glucose.

Blood-sugar determinations were performed on each fish used (total of 39) in this work immediately after its arrival in the laboratory from its place of capture in traps situated out in the ocean. The values obtained ranged between 72 and 250 milligrams of glucose per 100 cc. of blood. Sometimes very low values were recorded (from 0 to 50 milligrams per cent), but animals with such low blood-sugar figures were either moribund, or, if apparently normal, they invariably died within a few hours. In this respect our observations entirely agree with those of Scott (1921). After this initial blood-sugar determination, in order to let the fish recover from the effects of asphyxia and rough handling incident to its capture, it was placed in an immersed floating cage, several meters off shore, exposed to tides and marine currents. After 48 hours, a new sample of blood was withdrawn as quickly as possible and the amount of sugar determined. The figures thus obtained in 10 specimens of *Mustelus canis* ranged between 65 and 137 milligrams of glucose per 100 cc. of blood, the average being 105 ± 5 milligrams, and the standard deviation ± 22 milligrams. No food was given to the animals.

These results, as well as the data found in the literature regarding the blood-sugar level of elasmobranchs, seem to show a clear difference between the amount of glucose in the blood of the species of the genus *Mustelus* (smooth dogfish) as compared with that in the blood of species of the genus *Squalus* (spiny or horned dogfish). In effect, the "weighed average" of all the analyses on fish of the genus *Mustelus* reported by Fandard and Ranc (1914), Scott (1921), Menten (1927) and Fremont-Smith and Dailey (1932), turns out to be 99 milligrams of glucose per 100 cc. of blood, for a total of 34 animals examined. Denis (1922), omitting to say how many animals she investigated, reports for the blood of *Mustelus canis* amounts of glucose ranging from 80 to 181 milligrams per 100 cc., the majority of her results falling between 90 and 110 milligrams.

In so far as the genus *Squalus* is concerned, the "weighed average" of the analyses performed by Claude Bernard (1877), Lang and Macleod (1920), and White (1928), is of the order of 36 milligrams of glucose per 100 cc. of blood for a total of 15 animals examined. It is impossible to account for this difference on the basis of the view that the difference in the blood-sugar levels of these two genera is related to their different habits of life in the way pointed out by Gray and Hall for teleosts. While according to Bigelow and Welsh (1924), the spiny dogfish (*Squalus*) is a strong, fast-swimming animal, the smooth dogfish (*Mustelus*) is a bottom fish, feeding principally on crustaceans. Elasmobranchs of the genera *Torpedo* and *Scyllium*, which according to the analyses of Diamare (1905 and 1906), Diamare and Montuori (1907), and Kisch (1929) have also lower amounts of blood sugar than *Mustelus*, are slow-moving animals of the sea bottom (Couch, 1868).

Pancreatic Diabetes in Fish

Capparelli (1894) was apparently the first to study the effect of pancreatectomy on fish. He removed the pancreas from eels, and was able to find marked glycosuria as a consequence. Diamare (1905, 1906, and 1911), working on elasmobranchs, found considerable amounts of sugar in the blood of *Scyllium* and *Torpedo* after removal of the pancreas, whereas he was unable to detect any sugar in the blood of these animals before the operation. Probably Diamare and subsequently Diamare and Montuori (1907) failed to find sugar in the normal blood of *Scyllium* and *Torpedo* because of the inadequate methods available at that time, as they themselves suggested. More recently McCormick and Macleod (1925) found in *Myoxocephalus* (sculpin) marked hyperglycemia as a consequence of the ablation of the principal islets, easily removed in this animal, leaving intact the remaining pancreatic tissue. Simpson (1926) working on *Myoxocephalus* and *Ameiurus* confirmed the observations of McCormick and Macleod.

Experiments and Results

Limiting the present investigation to animals of the same species and following as uniform a procedure as possible in handling the animals before, during, and after the operations, it is possible to a certain extent to make "constants" out of the several factors, known and unknown, which modify the blood-sugar level, aside from the experimental conditions created for purpose of the study (pancreatectomy, hypophysectomy, etc.). Finally, a statistical treatment of the data obtained will enable us to get a more complete idea of the significance and validity of the differences between the average values obtained under the different experimental conditions (Dunn, 1929).

Medium-sized animals were chosen (from 70 to 90 centimeters long), regardless of sex, but pregnant females were rejected. Blood-sugar determinations were always performed by Shaffer and Hartmann's method (1921).

The first step of our procedure was always to withdraw a sample of blood from the heart as previously described, for a blood-sugar determination. Then the necessary operations were performed, with care to avoid asphyxia as much as possible. A constant flow of sea water was maintained through the mouth and gills and under these circumstances the respiratory movements proceeded in normal fashion.

The pancreas was removed through an abdominal incision about three centimeters long and the abdominal wall was subsequently closed in layers by silk sutures. The hypophysis was extirpated through a buccal approach. No anesthetic was used. The longest operation lasted about twenty minutes. After they had been operated the animals were treated as previously described.

Forty-eight hours after operation a new sample of blood was secured and another blood-sugar determination performed. The forty-eighth hour after operating proved to be a critical juncture.

TABLE I

Glycemia of Dogfish under Different Experimental Conditions
(Glucose in milligrams per 100 cc. of blood)

OPERATION	Laparotomy		Hypophysectomy		Pancreatectomy		Hypophysectomy and Pancreatectomy		Pancreatectomy and Injury of the Hypothalamus	
	Before operation	After 48 hours	Before operation	After 48 hours	Before operation	After 48 hours	Before operation	After 48 hours	Before operation	After 48 hours
Individual Results	83	150	144	100	185	431	165	155	115	431
	247	125	185	65	144	350	175	328	183	431
	220	115	170	370	144	428	115	267	203	430
	185	185	100	185	205	370	175	370	162	392
	160	105	160	95	124	431	225	205	144	423
	72	105	144	115	195	368	267	395		
	120	185	142	117	155	380	124	349		
	122	226	155	137	135	430	115	215		
					150	430	137	307		
Averages	151±13	149±11	151±6	148±23	159±6	402±7	166±11	288±18	161±10	421±5
Stand. dev.	±52	±42	±23	±90	±26	±32	±49	±77	±30	±15
Prob. error	±35	±28	±15	±60	±17	±21	±33	±52	±20	±10
Differences	2±16		3±23		243±9		122±21		260±11	

Before that time the effects of asphyxia and rough handling inherent in the operations were still too marked, and beyond the 48 hours the mortality began to be rather high.

The animals fall into five groups. The results show respectively the separate effects on blood sugar of (*a*) simple laparotomy, (*b*) hypophysectomy, (*c*) pancreatectomy, (*d*) pancreatectomy and hypophysectomy, and (*e*) pancreatectomy and injury of the hypothalamic region of the brain. The operations were performed in such a way as to permit having animals of different groups simultaneously exposed to the same environmental conditions.

As Table I shows, neither the laparotomy alone, nor the simple hypophysectomy, exerted any significant influence on the glycemia. The values, of course, were above those considered normal, but of the same order of magnitude as the values found before the operation. The removal of the pancreas, as was to be expected, caused a marked increase of blood sugar values: from 159 ± 6 milligrams of glucose per 100 cc. of blood as the average for 9 unoperated animals, to 402 ± 7 milligrams per 100 cc. of blood as an average for the same animals 48 hours after the operation.

When both pancreas and hypophysis had been removed in the course of the same operation, a condition of hyperglycemia also ensued, but the average for this whole group as well as the individual figures were lower than those encountered when the pancreas alone was taken out. That this difference (− 114 ± 19 milligrams) which is certainly significant, is due to the absence of the pituitary body and not to the influence of some direct nervous factor brought into play by the operative traumatism, is demonstrated by the fact that in animals of the fifth group in which the pancreas was removed, leaving intact the hypophysis but injuring the adjacent nervous tissue (hypothalamus), the blood-sugar values were even higher than those found when the pancreas alone was extirpated.

Shortage of time and animals prevented the study of the action of pituitary grafts and the action of pituitary extracts, but the data here reported support the conclusion that in the dogfish (elasmobranch fish) just as in the toad (batracian) or in the dog (mammal) the hypophysis exerts an aggravating influence on pancreatic diabetes, the mechanism of which is still obscure.

SUMMARY

Laparotomy or hypophysectomy does not change the blood-sugar level in the dogfish. Pancreatectomy produces a marked hyperglycemia. The hyperglycemia is, however, less marked if pancrea-

tectomy is accompanied by hypophysectomy, but it is slightly more marked if in addition to pancreatectomy the hypothalamus is injured.

In conclusion I wish to express my indebtedness to Dr. Philip Bard for valuable help and suggestions.

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MELANOPHORES INDUCED BY X–RAY COMPARED WITH THOSE EXISTING IN PATTERNS AS SEEN IN CARASSIUS AURATUS[1]

GEORGE MILTON SMITH

Anatomical Laboratory, School of Medicine, Yale University

In a recent publication (Smith, 1932) it was shown that if goldfishes (*Carassius auratus*) were exposed to X-rays, an eruption of corial melanophores occurred varying greatly in intensity in different fishes. In some fishes a general cutaneous melanosis resulted, leading even to death of the fish.

Generally speaking, the X-ray eruption of melanophores is a transient affair, appearing about the fifth day after exposure to X-ray or somewhat later. Newly formed melanophores increase rapidly in numbers to form pigmented areas often visible to the eye. By a process of degeneration, these melanophores disappear, and there results a restoration to normal coloring. At water temperatures of 70° F., depigmentation consumes roughly from two to four weeks, after which the cutaneous regions once more assume a normal color.

X-ray-produced melanophores in the goldfish behave, therefore, in much the same manner as melanophores produced in the same type of fish by trauma or in the healing of wounds or fractures as noted in earlier experiments (Smith, 1931).

It became of interest to learn what relationship, if any, melanophores newly produced from X-raying held toward groups of melanophores already existing in the goldfish in the form of cells massed to form a definite pattern of the body, head, or fins; and further, to note any evidence of degeneration in existing pattern cells following radiation.

Forty-five goldfishes, possessing various black patterns for the most part resembling those seen in Plate I, Fig. 1, were studied after exposure to 7 human erythema units of X-ray (sufficient usually to induce melanophores in the goldfish), and the behavior of melanophores in patterns and melanophores formed by X-raying were compared.

The technique of X-raying, found satisfactory in earlier experiments, was the following. The goldfish, anaesthetised in a solution

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[1] Aided by Grant from Blossom Fund.

of chloretone 1 to 2000 of water, was removed from this solution and placed on a folded towel directly under the X-ray tube with the entire left side facing directly upward. One unit of human erythema dose consisted of 100 k.p.v., 5 milliampere, 8-inch target-skin distance, no filter, 72 seconds exposure. Seven erythema units involved, therefore, an exposure of 504 seconds. After exposure, the fishes were kept under conditions of ordinary laboratory light in tanks of still water (70°–78° F.) supplied with a current of air. The X-raying was done through the courtesy of Dr. William LaField, Mr. E. E. Furbush of the New Haven Hospital, and Dr. Samuel Atkins of St. Mary's Hospital, Waterbury.

The following are two illustrative experiments, in which X-ray eruptions were intense enough to permit photographing.

Experiment 1. Goldfish, 5 cm. in length from snout to base of tail (Plate I, Fig. 1, Fish *A*, photographed before exposure to X-ray) with markings of massed melanophores on head and fins. This fish received 7 human erythema units of X-ray, exposing entire left side of fish. Eleven days later an active development of melanophores occurred in the exposed surfaces of the fish. Fourteen days after exposure the X-ray eruption appeared to have reached its height and the fish was photographed (Plate I, Fig. 2). Dense masses of X-ray-induced melanophores occupied chiefly the left side of head, body, and fins, and closely encroached upon the periphery of black pigmented patterns. Depigmentation of X-ray-induced melanophores began approximately 3 weeks after exposure. Plate I, Fig. 3, shows fish 27 days after exposure with depigmentation greatly advanced. The head region has cleared, leaving the pattern undisturbed. Complete disappearance of X-ray melanophores was noted on the fifty-fifth day after exposure. Plate I, Fig. 4, was taken on the seventy-ninth day after exposure and shows head pattern practically unchanged. The pigmented markings of the fins showed microscopically no apparent difference from the original arrangement as seen before X-raying.

Although melanophores composing pre-existing patterns remain usually undisturbed by exposure to X-rays in doses of 7 human erythema units, as in the above experiment, in three instances there seemed to be definite evidence of an induced degeneration of melanophores composing a pattern, and the following illustrative experiment describes such a degeneration of pattern following a typical X-ray eruption and depigmentation.

Experiment 2.—Goldfish measuring 6 cm. from tip of snout to base of tail received 7 human erythema units of X-ray, the entire left side of the fish being exposed to the X-ray tube. Plate II, Fig. 1, is a photograph of this fish 19 days after exposure, showing a marked massing of melanophores, *XR.*, on the left side of the head and operculum, approaching in distribution close to the small head pattern *P.* At the periphery of the head pattern, *P.*, a close intermingling occurred of both pattern cells, *P.*, and X-ray melanophores, *XR.*, seen in higher magnification in Plate II, Fig. 2. Degeneration of melanophore masses induced by X-ray began in the third week, and advanced by the twenty-seventh day to a complete clearing of the head region, the upper part of the operculum alone showing still massed X-ray-induced pigment cells, *XR.* (Plate II, Fig. 3). The head pattern, *P.*, at this time retained the details of its original form except at the caudal tip of the pattern where there was noted some degeneration of melanophores adjacent to an opaque zone (Plate II, Fig. 3, *O–O′*), a point where xanthophores had also disappeared. Plate II, Fig. 4, taken 42 days after exposure, shows the head pattern partly degenerated. Plate II, Fig. 5, 55 days after exposure, shows the head pattern no longer existing; and it was noted that the black pigmented markings on the various fins had disappeared to a very large extent. This particular fish, relatively sensitive to X-ray, showed beside melanophore degeneration, within the first two weeks a degeneration of xanthophores in several areas indicated in the photographs by the letter *O.* Zones of xanthophore degeneration appeared in life as streaky grayish opaque areas, confined chiefly to the left side of the body, which had been directly exposed to X-ray.

Ten fishes failed to give any eruption whatever of melanophores after exposure to seven human erythema units. A month later mechanical injury was produced by crushing the left operculum with an artery clamp. Five days later numerous melanophores developed near the wound in each fish, temperature of water being 76° F. It was believed that in these experimental fishes, X-ray injury was not severe enough to elicit a melanophore reaction.

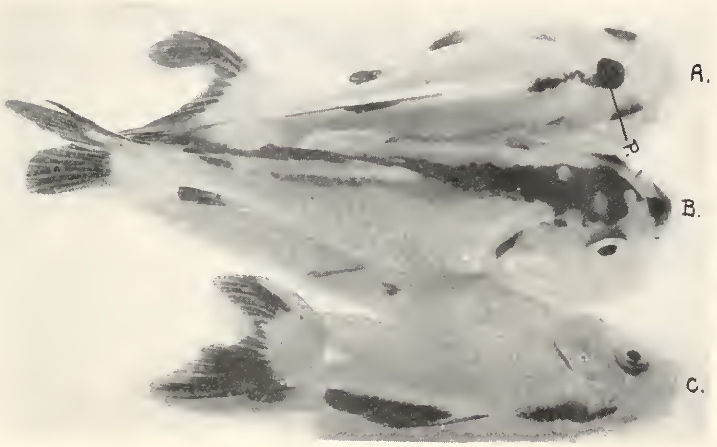
EXPLANATION OF PLATE I

FIG. 1, *A*, *B*, *C*, are types of goldfishes employed in these experiments, with black pigment patterns. Fish *A*, photographed on day before X-raying.

FIG. 2, Fish *A.*, 14 days after X-raying showing pigmentation from X-ray-induced melanophores (*XR.*) chiefly on left side or exposed side, encroaching upon region of existing pattern of head, *P.*

FIG. 3, Fish *A.*, 27 days after raying. Depigmentation of X-ray-induced melanophores greatly advanced, eruption showing only at points *XR.*

FIG. 4, Fish *A.*, Photograph shows fish *A* on the seventy-ninth day with head pattern intact. Complete disappearance of X-ray eruption occurred on the fifty-fifth day.



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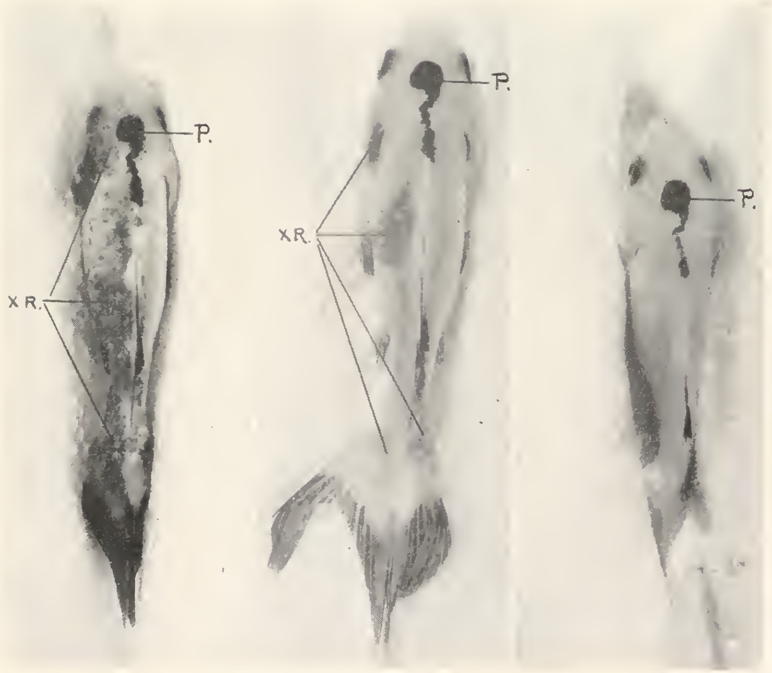

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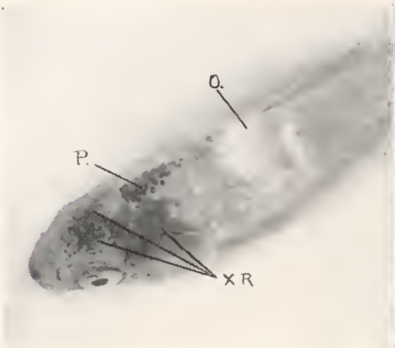
PLATE I



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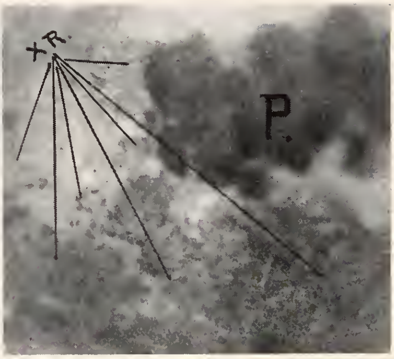

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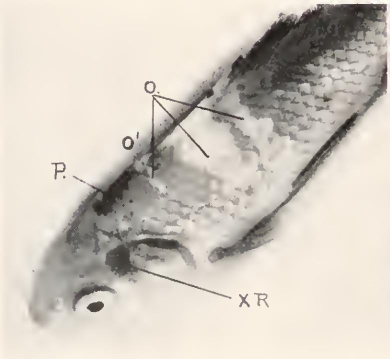

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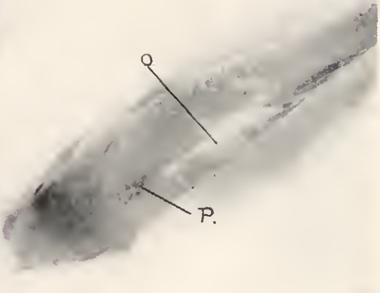

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PLATE II

Comment

Results of experiments indicated that melanophores of already existing black patterns were for the most part not influenced by single doses of X-ray as high as seven human erythema units. At the periphery of such patterns an active development of new melanophores from X-ray exposure might occur here and there, so that pattern melanophores and what may be designated X-ray melanophores grew in close apposition (Plate II, Fig. 2) with much intermingling at the time of the height of the X-ray eruption. Disappearance of the X-ray melanophore eruption left the melanophores of the pattern usually in an intact condition without alterations in the morphology of the cells. Thus, melanophores of two kinds were found to exist in the same fish, reacting differently to the effect of X-ray. On the one hand, melanophores composing existing patterns usually remained stabile, and rarely degenerated. On the other hand, masses of new melanophores evoked by X-ray, pursued a comparatively short life cycle of active growth and early degeneration with complete subsequent depigmentation.

Degeneration of pattern cells was noted definitely only three times among the 45 fishes studied, as illustrated in Experiment 2 cited above. As seen in this experiment, the head pattern slowly degenerated after disappearance of the X-ray-induced melanophore eruption. An extensive though incomplete degeneration of the fin patterns occurred simultaneously with that of the head pattern. In this particular fish, areas of degeneration of xanthophores were noted as well. It is not unlikely that further investigation will show that a dosage somewhat higher than 7 human erythema units will be neces-

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Explanation of Plate II

Fig. 1. Shows goldfish referred to in Experiment 2 with X-ray eruption (*XR.*) 19 days after raying. X-ray eruption (*XR.*) and head pattern (*P.*) are in close apposition. Letter *O* points to an area of xanthophore degeneration.

Fig. 2. A higher magnification of a part of the field in Fig. 1. *XR* points to eruption of massed melanophores induced by X-ray. *P.* represents the massed melanophores forming existing pattern of head. Melanophores of eruption and pattern intermingle at the periphery of pattern *P.* Magnification × 6.

Fig. 3. Same fish as in Fig. 1 with partial depigmentation of X-ray eruption of melanophores 27 days after X-ray exposure. The head region has cleared and shows no eruption of melanophores except at the upper part of the operculum (*XR.*) Pattern *P.* is intact except for slight degeneration posteriorly at *OO′* where xanthophores have also disappeared.

Fig. 4. Same fish 42 days after raying. Head is cleared of X-ray eruption; pattern *P.* is degenerating. Xanthophores have degenerated at point marked *O.*

Fig. 5. Same fish 55 days after raying. Head pattern has degenerated completely. Xanthophore degeneration at point marked *O.*

sary to produce uniformly degeneration of pattern melanophores of this fish, influenced by weight and size of fish.

The melanophores following X-ray exposure may bear a close morphological resemblance to the melanophores of an existing pattern, so that the two types are distinguished often with difficulty. Usually, however, X-ray melanophores look more delicate and smaller than pattern melanophores; their processes are more irregular and reach out into different planes in the tissue spaces. The pattern melanophores appear more flattened as they lie at rest spread out immediately beneath the transparent epithelium. Their borders with processes parallel to the surface appear more sharply circumscribed and deeper pigmented especially when in a somewhat contracted state.

As Fukui (1927) and Goodrich and Hanson (1931) have shown, the young goldfish is normally dark colored as the result of the presence of melanophores. Depigmentation begins irregularly after a few weeks of life and the fish gradually assumes a yellowish, golden color. The extent and completeness of depigmentation determines the pattern of adult conditions, subject probably to still further slow changes in black pigmentation later in life. Melanophores of patterns are probably fully differentiated cells, and closely affiliated with the nervous system as shown by Ballowitz (1893), von Frisch (1911) and other investigators; whereas melanophores evoked by X-ray, or by mechanical injury function perhaps in behalf of body defense and repair, when certain chemical conditions are produced in the corium of goldfishes possessing potential pigment-forming cells.

In the present experiments, areas composed of pattern cells did not seem to develop new X-ray-induced melanophores to any extent except temporarily at the periphery of the pattern. This fact suggests that conditions did not exist in the central parts of the patterns for the development of new pigmented cells, under the conditions of dosage employed, or possibly that the massed flattened pre-existing pattern cells offered enough protection against the effects of X-rays to inhibit the formation of new pigmented cells.

SUMMARY

In the goldfish exposed to X-ray (7 human erythema units) existing patterns remained for the most part intact in the presence of an induced temporary eruption of corial melanophores caused by X-raying. In several fishes, however, a degeneration and disappearance of the patterns, partial or complete, was noted, and this followed after depigmentation of an eruption of X-ray-induced melanophores. X-ray thus produced two effects relative to melanophores, (*a*) an eruption of new melanophores with a short life cycle, (*b*) occasional degeneration of melanophores in existing patterns.

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INTRACELLULAR CRYSTALLIZATION OF HEMOGLOBIN IN THE ERYTHROCYTES OF THE NORTHERN PIPEFISH, SYNGNATHUS FUSCUS

ALDEN B. DAWSON

(*From the Zoölogical Laboratories, Harvard University, and the Marine Biological Laboratory, Woods Hole, Mass.*)

It has long been recognized that the hemoglobins of different animals vary widely in solubility and ease of crystallization and that hemoglobin rarely crystallizes within the red blood corpuscles. In a previous paper (Dawson, 1930) intracellular crystallization of hemoglobin was described for the erythrocytes of the urodele, *Necturus maculosus*. In this case crystallization was apparently favored by previous poisoning with lead acetate, although it had been occasionally encountered in normal animals.

A similar phenomenon has been observed in the erythrocytes of the northern pipefish. The animals were obtained in the Eel Pond, at the Marine Biological Laboratory, Woods Hole, and appeared to be normal in all respects. The observation was made incidentally while studying supravitally the blood cells of the common marine fishes of that locality. The crystallization of hemoglobin in the erythrocytes of the pipefish is readily induced by slowly drying, in the air, rather thick smears of blood, and is most uniformly obtained when the humidity is relatively high. Preceding the appearance of definite crystals the cells lose their typical oval form and show an increasing tendency towards angularity. The majority finally assume a triangular shape but some become rhomboidal. However, at this stage the hemoglobin gives no evidence of crystal formation. Soon well-defined clefts appear in the cell contents and definite crystals then appear.

The number of crystals formed in individual cells is subject to some variation. Three crystals, forming the three sides of a triangle with the nucleus in the center, are most commonly encountered. Occasionally four crystals forming a rhomboidal figure, and more rarely two crystals arranged parallel with the long axis of the cell are present (Fig. 1). Frequently a variable number of very small, slender crystals may be associated with larger ones. They usually lie irregularly about the nucleus. The size of the larger crystals is also somewhat variable, but the shape is relatively constant. Practically all are modified on the side next the cell membrane, being rounded rather than straight. In addition many are notched on the inner side especially if they are in contact with the surface of the nucleus.

DISCUSSION

Little is known of the factors involved in maintaining the hemoglobin within the red blood cell in solution. In the present instance the only obvious cause of the crystallization of the hemoglobin in the erythrocytes of the pipefish is the slow withdrawal of water with whatever attendant injuries that may occur when drying takes place. It is of interest to note that the cell is deformed by the changed orientation of the hemoglobin molecules before any change in the nature of the hemoglobin can be observed with ordinary transmitted light and that the form acquired by the cell, triangular or rhomboidal, foreshadows the appearance of three or four major crystals within it. Moreover, the clefts which mark the amount of hemoglobin to be apportioned to each crystal also become evident while the hemoglobin still appears unmodified. One of the striking features of all erythro-

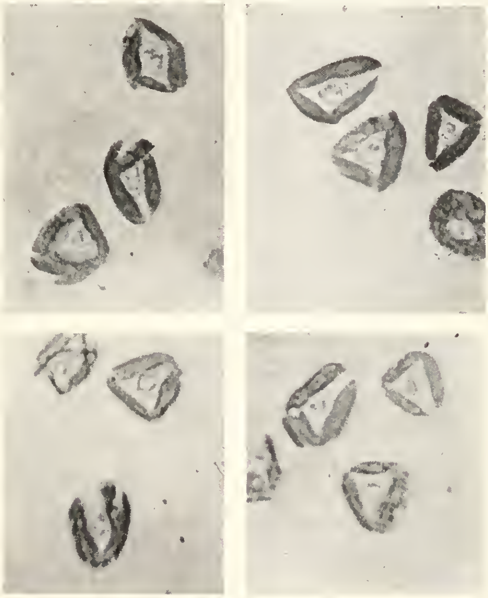

FIG. 1. Four selected areas from a preparation of pipefish blood showing the characteristic numbers, size, form, and position of hemoglobin crystals within the erythrocytes. The turbidity of the background is caused by the laking of many cells due to the injury produced in transferring the cells from the slide to a coverslip in order to obtain a preparation thin enough to photograph. Magnification × 1150.

cytes is their tendency to return to their specific form after deformation, but in the case of incipient crystallization the shift in orientation of the hemoglobin molecules is sufficient to produce a permanent distortion.

In the case of *Necturus*, previously described, and in the pipefish the crystals of hemoglobin are large and relatively few. In other instances that have come under my observation while studying supravitally the erythrocytes of many vertebrates, the crystallization of hemoglobin has been quite different, the crystals being numerous and very small, producing a granular effect. Such crystallization has been encountered on a few occasions in *Necturus* as well as in another urodele, *Eurycea bislineata*. It has also been noted in several fishes such as the common mackerel, menhaden, alewife, and sea bass. In all of these cases the cause of the crystallization was unknown and appeared irregularly in preparations of fresh blood.

In a review of the literature one finds few references to intracellular crystallization of hemoglobin. Guerber (1927) observed it in the erythroblasts of embryos of the pig and cow. Kranz (1928) described crystals in mammalian erythrocytes after fixation with potassium bichromate and acetic acid, followed by paraffin imbedding. Celloidin imbedding gave negative results. He believed that the crystals were not pure hemoglobin but a product resulting from the reaction of hematin with the chromic and acetic acids. The work of Kranz was subsequently repeated by Tschachmachtschian (1932) who concluded that the crystals described by Kranz were entirely an artefact, the result of paraffin imbedding, and were not directly related to the hemoglobin content of the erythrocytes.

Jokl (1925), while studying fresh preparations of skate's blood, observed certain erythrocytes in which the cell content was divided obliquely by two or three peculiar light stripes. These light stripes appear comparable to the clefts which appear in the hemoglobin of the red cells of the pipefish, preceding the appearance of the large crystals.

Intracellular crystallization of hemoglobin was encountered in certain teleosts by Yoffey (1929), although he failed to recognize it as such. He states: "In the *Gadus* group the erythrocytes may assume a very curious shape. At first round, they then become oval, as in other fishes. They then show an increasing tendency towards angularity, and finally may become perfectly triangular in shape (Fig. 15). The relative proportion of triangular to oval red blood corpuscles varies from one animal to another. The illustration shown is from a blood film of *Gadus minutus* in which the majority of the erythrocytes are triangular. On the other hand there are many cases in which only a few of the corpuscles are triangular, and the majority are of the normal shape. The triangularity is not artificially produced by the fixative because it may be observed in specimens of perfectly fresh and unfixed blood, though the angles may not be sharp as in the fixed film." (p. 336.)

From Yoffey's description it is obvious that in *Gadus minutus* he was dealing with intracellular crystallization and, in the photograph reproduced in his Fig. 15, three large crystals are clearly seen in almost every cell. Apparently in the *Gadus* group crystallization of hemoglobin occurs as readily as in the pipefish and was induced by Yoffey unconsciously by slight variations in his technique.

SUMMARY

Crystallization of hemoglobin within the erythrocytes of the pipefish is described. This phenomenon is readily produced by slow drying, especially in a humid atmosphere.

Preceding the appearance of the definitive crystals the erythrocytes lose their characteristic oval form and become angular, triangular and rhomboidal forms predominating. Then definite clefts appear, followed soon after by the appearance of typical crystals. The number of crystals within individual cells varies. Two, three, and four large crystals are most commonly encountered, but a more variable number of minute needle-like forms may also be present in the erythrocyte.

It is of interest to note that the erythrocytes exhibit deformation due to the changing orientation of the hemoglobin molecules before any evidence of crystal formation can be detected with ordinary transmitted light.

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SALT REQUIREMENTS AND SPACE ORIENTATION OF THE LITTORAL ISOPOD LIGIA IN BERMUDA[1]

T. CUNLIFFE BARNES

Osborn Zoölogical Laboratory, Yale University

Introduction

The important problem of the invasion of terrestrial or fresh-water habitats by marine organisms has received considerable attention in recent years (cf. Schlieper, 1929; Pearse, 1929; Pantin, 1931). The Isopoda extend from great depths of the ocean to terrestrial and fresh-water environments and should afford especially interesting material for these investigations. Tait (1916) in particular has studied the longevity of the littoral form, *Ligia oceanica*, in dilutions of sea water. The structure of *Ligia* has been described in detail in the monograph of Hewitt (1907).

The present paper deals chiefly with the effect of changes in the salt content of sea water on *Ligia baudiniana* Milne-Edwards, the common isopod in Bermuda.

Habits

Ligia baudiniana may be found in great numbers on the rocky shore (Verrill, 1903), especially in the intertidal zone at low tide. *L. oceanica* (Tait, 1916) sometimes remains covered with the tide but *L. baudiniana* retreats as the water advances. When isolated on stones in tidal pools, the isopods will run from one side of the rock to the other as if seeking a dry pathway to the shore. Occasionally I have observed them leaping from one stone to another to avoid the water. However, they are dependent on the sea water to keep the surface of the gills moist and we have never found specimens more than seventy feet from the sea; they appear in great numbers on rocks and walls several feet from the sea on rainy days. If placed in a terrarium containing a small pool of sea water, they will crawl to the edge of the water, turn around, and dip the ends of the uropodal spines in the water. By bringing the spines close together and altering the distance between the spines in a slow rhythm, the isopod moistens the gills with the water which rises between the spines by capillarity (Fig. 1). In this way a large drop of water may form on the gills.

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[1] Contribution from the Bermuda Biological Station for Research.

These spines, which are usually long, are also used as swimming fins and feelers as in other species of *Ligia*.

It is probable that *Ligia* enters the sea to release the young from the brood pouch for this occurred only in submerged specimens.

An examination of the gut contents revealed vegetable debris and unicellular algae. According to Hewitt (1907) *L. oceanica* feeds largely on decaying vegetable matter. *L. exotica* is described as omnivorous by Pearse (1931). Although Pearse (1929) is undoubtedly correct in pointing out that food supply alone is probably not the most important factor in determining the habitat of littoral forms, yet it is of interest to note that *Ligia baudiniana* subsists largely on the green coating of rocks in the intertidal zone.

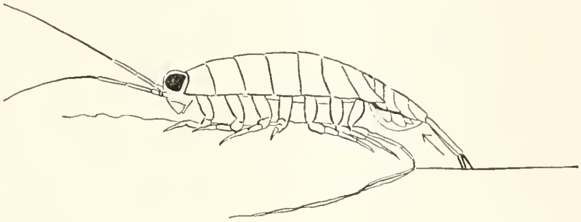

FIG. 1. *Ligia baudiniana* wetting the gills by the capillary action of the uropodal spines which are dipping into the sea.

CONCENTRATION EFFECTS

As a basis for comparison for subsequent experiments, the longevity of the isopods was first determined in sea water, air, and in fresh water. Individual specimens, carefully collected to avoid injury, were placed in finger bowls containing 100 cc. of water or solution. The average duration of life was only four hours in distilled water, seven and one-half hours in fresh water and thirty-four hours in sea water (Table I). The maximum longevity is also given in the tables. Sea water, changed every twenty-four hours, gave an average duration of life of fifty-eight hours, and in running aerated sea water the average longevity was one hundred and ninety-two hours, but the maximum was recorded for unchanged sea water. The large surface exposed to the air in the finger bowls permitted considerable diffusion of O_2 and CO_2 as is indicated by the maximum of twelve and one-half days in unchanged sea water. In dry glass dishes the average duration (and the maximum) was eleven hours, but in bowls containing damp sand the isopods lived for very long periods (fifteen days). It is clear that moist air is a far more favorable medium than sea water. *Ligia* dies in about one hour in dry air in the sun at 30–37° C. In sea water the isopods survive overnight at a temperature of 5° C., but the gills cease beating at 15° C.

The life of *Ligia* in sea water is markedly curtailed by dilution below 50 per cent or by doubling the concentration of salts (Table II). In dilute sea water there is a slight increase in the frequency of gill movements. The brief existence of the isopod in distilled water is not due to the decreased osmotic pressure, for the addition of glycerine has little beneficial effect (Table II). On the other hand, the death of *Ligia* in concentrated sea water appears to be due largely to osmotic factors as will be seen in the records of rapid death for sea water in which the osmotic pressure was increased by glycerine.

TABLE I

Longevity of Ligia in Air and in Water

Medium	Average Duration of Life	Maximum	No. of Specimens Tested
	hours		
Distilled water	4	5	21
Fresh water	7½	8	6
Sea water	34	297	93
Sea water (changed daily)	58	198	20
Running sea water	83	192	26
Air	11	12	8
Air over damp sand	360	625	31

TABLE II

Concentration Effects on Ligia

Medium	Average Longevity	Maximum	No. of Specimens
	hours		
25% sea water	6	10	6
50% sea water	20	70	7
75% sea water	51	172	8
200% sea water	3	7	5
250% sea water	1	1	4
400% sea water	1	2	3
M/2 glycerine	7	10	5
M glycerine	5	12	13
M/4 glycerine in sea water	11	24	8
M/2 glycerine in sea water	9	19	6
M glycerine in sea water	4	5	6
2 M glycerine in sea water	2	2	3

SPECIFIC ION EFFECTS

Of solutions containing a single salt isosmotic with Bermuda sea water (5/8 M), the isopods lived longest in NaCl, eight hours, and $CaCl_2$, seven hours; while the average duration of life in $MgCl_2$ and KCl was only four and one and one-half hours respectively (Table III). KCl exerted an immediate paralyzing effect on the gills which normally began to vibrate as soon as the animal was immersed in any of the solutions mentioned in this paper except KCl. The frequency of gill movements was taken at intervals in all solutions, but no vibrations were ever observed in KCl although the animals appeared quite normal for the first half hour. The average time for ten beats was three and one-fifth seconds in sea water (27°); and approaching death was indicated when this increased to five seconds. Immature specimens (12–15 mm.) had a faster rate, two and one-tenth seconds, and were not used; in addition it was found that immature specimens showed greater resistance to all solutions tested.

TABLE III

Specific Ion Effects on Ligia

Solution	Average Length of Life	Maximum	No. of Specimens
	hours		
5/8 M NaCl	8	14	12
5/8 M $CaCl_2$	7	13	19
5/8 M $MgCl_2$	3	5	7
2.5/8 M $MgSO_4$	4	4	10
1 M $MgSO_4$	3	4	8
5/8 M KCl	$1\frac{1}{2}$	3	10

IONIC ANTAGONISM

Combinations of two ions were tried in various proportions, but no satisfactory solution was found. No specimens lived for more than a very few hours in binary mixtures but some antagonism seemed evident between Na and Ca. In solutions containing Mg or K the longevity seemed to be controlled by the amount of the most toxic ion present. In artificial sea water (*i.e.*, 100 NaCl, 11.6 $MgSO_4$, 2.2 KCl and 2.5 $CaCl_2$ in 5/8 M conc.) the duration, forty hours, compared very favorably with natural sea water. In artificial sea water containing no magnesium, the same average duration of life was exhibited (Table IV). If the KCl was omitted, the isopods lived for twenty hours; in the absence of Ca, fourteen hours; and they died within two hours in artificial sea water containing no Na.

The next step was to determine how long *Ligia* would survive if the concentration of individual ions were increased in sea water. In the case of Na, Ca and Mg concentrations not exceeding M/8 made up in sea water (*i.e.*, one-eighth of the molecular weight added to a liter of sea water) did not exert an appreciable toxic effect but KCl showed a limiting concentration of M/10 in sea water (Table V).

TABLE IV

Longevity of Ligia in Antagonistic Solutions

Solution	Average Length of Life	Maximum	No. of Specimens
	hours		
Artificial sea water	40	123	8
Same without Na	2	2	5
Same without Ca	14	23	10
Same without K	20	28	9
Same without Mg	41	120	22

TABLE V

Effect of Increasing Concentration of Ions in Sea Water

Solution	Average Longevity	Maximum	Number of Specimens
	hours		
5/8 M NaCl made in sea water	9½	36	10
1/4 M NaCl made in sea water	11	27	5
1/6 M NaCl made in sea water	6	11	8
1/8 M NaCl made in sea water	57	244	5
2.5/8 M $CaCl_2$ made in sea water	1½	2½	9
1/8 M $CaCl_2$ made in sea water	88	248	8
2.5/8 M $MgCl_2$ made in sea water	3	6	5
1/8 M $MgSO_4$ made in sea water	91	258	5
5/8 M KCl made in sea water	2	3	13
1/4 M KCl made in sea water	3½	5	9
1/6 M KCl made in sea water	5	5	9
1/8 M KCl made in sea water	6½	16	9
1/10 M KCl made in sea water	31	201	14

The gills were completely inhibited in 5/8 M KCl in sea water but exhibited the usual rhythm in M/4 KCl in sea water. In all these solutions in which salts were added to sea water it is probable that the increased osmotic pressure was significant judging from the short life of isopods in sea water containing glycerine (Table II).

SPACE ORIENTATION

Ligia baudiniana inhabits a very restricted zone along the shore line, and a number of experiments were performed to discover what tropisms or other reactions restricted the distribution of the isopod. One may mention first the inability to survive in sea water or in dry air and the presence of food (unicellular algæ) on the intertidal rocks. These facts, however, do not explain the curious ability of the animal to orient towards the sea when released a short distance from the shore. It was noted that the isopods appeared to be reacting to the inclination of the land sloping gradually to the sea and it was found that under controlled conditions (in a photographic dark room under dim red illumination) pronounced geotropic orientation was exhibited.

Table VI

Orientation of Ligia on Slopes near the Sea

Date	Temp.	Direction of Slope	Inclination	Number Released	Direction of Creeping
	° C.		*degrees*		
July 10	27	Towards sea	30	6	5 down
11	27	Towards sea	40	2	down
11	27	Away from sea	40	2	down
Aug. 1	26	Towards sea	40	9	7 down 1 up 1 went up but turned
12	27	Away from sea	30	6	5 down
14	25	Towards sea	40	5	down
14	25	Right angles to sea	60	4	3 down
14	25	Away from sea	20	4	3 down 1 up
14	25	Away from sea	45	4	2 straight down 3 down obliquely
24	28.6	Away from sea	60	4	3 down
25	27	Towards sea	50	3	down
25	27	Away from sea	20	5	4 down
25	27	Away from sea	30	10	4 down 6 up

To test this hypothesis, specimens were released at various distances from the sea on ground (sand, grass, or rocks) sloping towards and also away from the sea (Table VI). Of sixty-four isopods tested, fifty showed positive geotropic orientation and crawled downward regardless of the direction of the sea. They also showed a less pronounced tendency to crawl in the direction of open patches of sky and exhibited positive phototropism under controlled conditions.

When isopods are released in the sea at a distance not greater than eight feet from shore, they swim energetically to shore or crawl over the bottom directly to shore. In general, the animal crawls over the bottom and seldom exhibits the typical swimming movements unless in deep water. The cause of this orientation to shore in the sea is unknown; it is independent of currents or the direction of the sun. The animal is negatively rheotropic and will swim against currents in an aquarium even after the removal of antennæ and uropodal spines, but this has nothing to do with the shoreward orientation. Luther (1930) has shown recently that the antennules of crabs are receptors for rheotropism, but in *Ligia* the antennules are extremely small (cf. Hewitt, 1907), and it seems probable that currents in the water stimulate the legs.

DISCUSSION

Ligia affords a striking example of a marine organism which is invading the land through the intertidal zone—an approach to terrestrial life which has not received sufficient attention in theoretical considerations of the evolution of land animals. In tropical islands, lacking fresh water, and where there is no pronounced temperature difference between sea and air, the intertidal zone becomes an important route for the invasion of the land (Pearse, 1929). *Ligia baudiniana* contrasts sharply with *L. oceanica* which, according to Tait (1916), may live over eighty days in sea water. However, like the beach crab *Ocypode*, *L. baudiniana* is dependent on sea water to keep the gills moist for aerial respiration. It also resembles the beach crab in its inability to withstand fresh water or diluted sea water, and is thus quite different from the marine *Gammarus*, which lives long periods in sea water diluted to .5 per cent (Adolph, 1925). The death of *Ligia* in distilled water and in glycerine solutions indicates that, like *Gammarus* (Loeb, 1903), loss of essential salts is more detrimental than osmotic disturbances in the medium. In spite of its terrestrial life, *Ligia baudiniana* is clearly a poikilosmotic form, although in diluted sea water the respiratory rate increases, which on Schlieper's (1929) theory might be due to osmotic work performed in partially resisting disturbance of the water and salt equilibrium.

The order of toxicity of single ions, $K > Mg > Ca > Na$, appears to be about the same for several Crustacea, *i.e.*, *Gammarus* (Adolph, 1925), *Artemia* (Martin and Wilbur, 1921), *Daphnia* (Berger, 1929), *Cambarus* (Helff, 1929), and is the reverse of the toxicity series for the egg of the sea urchin (Page, 1929). The rapid death in KCl is probably due in part to the lack of ventilation of the gills, which are unable to move in this solution. Zoond (1931) has shown that ventilation of the gill surface is of vital importance in Crustacea due to the extremely slow rate of diffusion of O_2 in water. The recent experiments of Bialaszewicz (1932) have demonstrated that the high toxicity of KCl for Crustacea is associated with its rapid disappearance from the blood nto the tissues. According to Loeb (1903) Na, K, and Ca are necessary for the gill movements of *Gammarus*, but it resembles *Ligia* in certain other salt requirements, *i.e.*, there is no satisfactory binary mixture and Mg appears to be a dispensable ion.

The orientation of *Ligia* to the sea resembles that of young loggerhead turtles described by Parker (1922). The orientation of the isopod to the shore, when in the sea, appears to be an instance of definite orientation which is not governed by a simple type of tropistic response and is not unlike the orientation of *Onchidium* (Arey and Crozier, 1921), and of ants (Barnes, 1929). The fact that the orientation of *Ligia* in the sea is not exhibited at distances greater than ten feet from the shore precludes the possibility that the isopod reacts to the blue color of deeper water as in the loggerhead turtle (Hooker, 1910).

SUMMARY

1. *Ligia baudiniana* moistens its gills by the capillary action of the uropodal spines.

2. The isopod survives best in damp air and is unable to live for long periods in sea water.

3. Changes in the concentration of sea water are detrimental, *i.e.*, *Ligia* is poikilosmotic.

4. The cations of sea water are toxic in the following order: K > Mg > Ca > Na. K exerts a specific paralyzing effect on the gill movements.

5. On land *Ligia* orients toward the sea. Positive geotropism appears to be the most important factor.

I am greatly indebted to my co-worker, Mr. Frank Gilchrist, who performed most of the early experiments.

It is a pleasure to express my gratitude to Dr. J. F. G. Wheeler who placed the facilities of the Bermuda Station at my disposal.

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THE RELATION BETWEEN ABSORPTION AND ELIMINATION OF WATER BY TERMOPSIS ANGUSTICOLLIS

S. F. COOK AND K. G. SCOTT

(From the Division of Physiology, University of California Medical School, Berkeley, California)

The purpose of this investigation was to study the water relations of the termite from the point of view of the animal itself. The question has been of interest in its ecological aspects with reference to the climatic conditions favorable for termite growth and to moisture and dryness as factors determining the distribution of species. As far as we are aware no studies have been undertaken to determine under controlled conditions from what source the termite derives its water, how it undergoes water loss, and how much drying it can suffer without ill effect. These questions are considered in the data here presented.

The organism used was *Termopsis angusticollis*, the common large wood termite of the Pacific Coast. The termites were collected in the vicinity of Berkeley and kept in jars of moist rotten wood until needed. The first step was to determine the normal water content of *Termopsis* and establish the relation between the partial pressure of the water vapor in the atmosphere surrounding the termites and their water content.

Experiment 1. Three groups of 50 termites each were placed in desiccators. In the desiccator with Group I was placed a vial containing water. At equilibrium, therefore, the relative humidity was 100 per cent and the partial pressure of water vapor from 15 to 17 mm. Hg, since the temperature varied from 17° to 20° C. It was not possible to maintain an absolutely constant temperature, but the variation of approximately 3° was too slight to affect the validity of the result.

In the second group the vapor pressure was lowered to 7 to 9 mm. Hg, the variation again being dependent on the temperature. This was accomplished by placing in the desiccator with the termites a saturated solution of calcium chloride over solid calcium chloride. The vapor pressure should theoretically be of the order of magnitude mentioned. This was checked by evacuating a vessel containing a similar mixture of solid and dissolved calcium chloride and allowing the system to come to equilibrium, the actual pressure of the water vapor being read on a mercury manometer. The use of this mixture is also of advantage since the absorption of water from the termites does not alter the concentration of the dissolved calcium chloride when the solid phase is present in excess. Since the termites were transferred directly from conditions of water saturation to this tension, any effects due to the reduced tension should become immediately apparent.

The third group of termites was placed in a desiccator with anhydrous calcium chloride where the vapor tension was less than one millimeter.

All three groups of termites were kept under the conditions observed above for two days. Food was provided in the form of punky wood which had been allowed in each case to come into equilibrium with the appropriate vapor tension before the experiment began. Starvation, therefore, was eliminated as a factor in reducing the weight of the termites. The termites were weighed before and after the exposure to the different degrees of humidity and the results expressed as average weight per termite. This was permissible because there was a sufficiently large number of animals in each case (50) to justify an average. Finally the dry weight was determined. The data are summarized in Table I.

Table I

Water loss by termites at different vapor tensions. In each case the initial number was fifty.

A.	Group I	Group II	Group III
B. Vapor tension in mm. Hg	15–17	7–9	0–1
C. Average fresh weight in mg	42.4	29.25	34.5
D. Average weight after experiment in mg	39.8	24.8	26.3
E. Average dry weight after experiment in mg	11.1	5.9	8.0
F. Water loss in per cent: $\frac{C - D}{D} \times 100$	6.2	15.3	23.1
G. Water content of termites at beginning in per cent: $\frac{C - E}{C} \times 100$	73.8	79.9	76.8
H. Water content of termites at end of experiment: $\frac{D - E}{C} \times 100$	72.2	76.1	69.6

From this table it is evident that the percentage of water lost is inversely proportional to the water vapor tension. Furthermore, the water content and the average weight appear to stand in inverse ratio. This probably can be explained by the fact that the older insects have developed wing pads and a thicker integument, thus lowering the relative water content. Nevertheless the extreme variation in relative water content which may be ascribed to this factor is of the order of 6 per cent (Line *G*, Table I), whereas the extreme observed variation is approximately 17 per cent (Line *F*, Table I). There is little doubt, therefore, that the water loss is substantially proportional to the decrease in vapor tension. It is highly probable that most of this water is lost through evaporation from the tracheæ and the body surface.

Experiment 2. In order to check the above results, an experiment was performed in which the water actually given off by the termites was compared with the loss in weight of the animals. Fifty termites were placed in a test tube through which dry air was passed for 212 hours. Before reaching the animals the air was dried thoroughly by passing it through ten feet of one-inch glass tubing filled with anhydrous calcium chloride and then through a flask of pure, concentrated sulphuric acid. The water and carbon dioxide given off by the termites was absorbed by anhydrous calcium chloride of the finest mesh obtainable, packed tightly in a medium-sized U-tube. Since the total quantity of carbon dioxide and water produced was small, and since the rate of flow was very slow, there can be little doubt that the absorption by the calcium chloride was practically complete. The carbon dioxide and water were not determined separately because the average carbon dioxide production of *Termopsis* has already been determined by one of us (Cook, 1932). According to the data presented in the paper mentioned, the average production is approximately 8.9 milligrams per gram termite per hour. The calculated value of the carbon dioxide produced during any time interval could then be deducted from the gain in weight of the calcium chloride with the reasonable assurance that the balance represented water. The initial weight and that at the end of the experiment were obtained, also the weight of food consumed (filter paper) and the weight of the feces (see Table II). Under the conditions described, viz., a very slow current of air, the water loss proceeded at a fairly regular but diminishing rate until the effects of the drying were apparent in the behavior of the termites. The effect of rapid drying is described elsewhere.

The total gain in weight of the calcium chloride in 212 hours was 628 milligrams, of which 150 milligrams (0.369 milligram per hour x 212 hours x 1,910 milligrams) may be ascribed to carbon dioxide, leaving 478 milligrams as the weight of the water. Meanwhile the loss in weight of the termites was 369 milligrams.

The excess water found may be accounted for in two ways. In the first place, since the diet was almost exclusively cellulose, and had been for weeks previous, and since the R.Q. under such conditions is very nearly unity (Cook, 1932), the predominant oxidation must have been that of carbohydrate. This should, of course, yield one molecule of water to each molecule of carbon dioxide, and if the total weight of carbon dioxide was 150 milligrams, as suggested above, the corresponding weight of the water produced would be 61 milligrams. In the second place, the three termites which died and were eaten must have contained, on the basis of the data presented with Experiment 1, about 75 per cent of water, or 90 milligrams. This water must have appeared in the calcium chloride tube directly by evaporation from the three termites while living or dead, or have been consumed by the others. In the latter case it would have been lost again from the surviving animals before the completion of the experiment. These two sources of water combined would furnish, therefore, a maximum of 150 milligrams, which when subtracted from the total of 478 milligrams found equals 328 milligrams. This is comparable with the net loss in weight of 369 milligrams.

TABLE II

Water Loss by Fifty Termites in a Slow Current of Dry Air

Initial total weight	Final weight	Filter paper consumed	Feces	Water lost in indicated intervals	Total water lost at end of indicated periods
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
2,030	1,541	105	92	83 in 19 hrs.	83 in 19 hrs.
				83 in 23 hrs.	165 in 44 hrs.
				43 in 14 hrs.	208 in 58 hrs.
				29 in 10 hrs.	236 in 68 hrs.
				47 in 24 hrs.	283 in 92 hrs.
				78 in 48 hrs.	360 in 140 hrs.
				77 in 48 hrs.	438 in 188 hrs.
				40 in 24 hrs.	478 in 212 hrs.

The water loss obtained in Group III of Experiment 1 was 23.1 per cent. In this experiment the loss as based on the water found in calcium chloride is 19.3 per cent, and as based on the loss in weight is 17.2 per cent. The results obtained by the two methods of estimation in Experiment 2 show a reasonably close correspondence. The difference between the degree of water loss in Experiment 1 (23.1 per cent) and in Experiment 2 (19.3 and 17.2 per cent) is not sufficient to invalidate the conclusion that under prolonged conditions of dryness the termites lose water to the extent of approximately 20 per cent of their weight.

Experiment 3. The water loss having been shown to be primarily through evaporation, the mode of intake was next investigated. There are but two possibilities. The water must be absorbed from the atmosphere or taken in with the food. If the termite can use atmospheric water, it must be able to absorb the vapor from a fully saturated atmosphere. If it can supply its needs adequately in this way, it should be able to utilize very dry food. If it cannot, then at least some water must be presented in the liquid form with the diet. To differentiate between these two possible sources of water, three groups of 25 termites each were kept under different conditions of atmospheric and food moisture. All three groups were fed wood which had been dried in a desiccator over calcium chloride for several days prior to the experiment. Owing to the great power of cellulose to absorb water even from the most powerful drying agents known, the water content could not have been zero, but it was lowered sufficiently to demonstrate the inability of the termite to live on relatively dry food.

It is obviously impossible to feed termites dry wood in a saturated atmosphere or damp wood in a completely dry atmosphere. Therefore the separation of food and atmospheric water had to be made in *time*. This was done as follows: Group I was kept 22 hours per day in saturated air without food, and was placed in a desiccator with dry air and dry food two hours. Group II was exposed to moist air 16 hours and to dry air and food 8 hours. Group III was exposed to each set of conditions 12 hours per day. In no case could the termites get much water from the food, but if it is possible for them to utilize dry food and atmospheric water, then at least Group I should be able to absorb as much water in 22 hours at a high humidity as it would lose during two hours at low humidity, and thus the normal water content should be maintained. But if they are wholly dependent on food moisture for their water intake, then all the groups might be expected to lose water irrecoverably during the period of exposure to dryness.

The water loss was determined by weighing after four days, but the termites were kept under the same conditions until they died in order to secure information concerning their viability. The data are presented in Table III. It will be observed that the water loss in all groups is substantially the same and is of the order of magnitude observed in the two previous experiments (20 to 23 per cent).[1] This seems to be the case even though the periods of feeding varied so that in Group I the termites had only two hours to lose water and twenty-two in which to regain it. But they lost as much water in four feedings, or a total of eight hours, as Group III, which was placed in dry air six times as long.

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[1] The effect of starvation in four days may be neglected, particularly in view of the fact that in Experiment 2 it was shown to play no significant rôle even in eight days.

The substantially equal loss of water with such widely varying total time of exposure to dryness as 8 and 48 hours presents an apparent anomaly. But this anomaly in itself shows that the water loss in dry air and dry food is *cumulative* and that intervening periods in damp air of as long as 22 hours per day do not tend to decrease the entire water loss incurred during the combined time of the dry feeding periods, even though this total time is as short as 8 hours. In other words, after losing water during a short exposure to dryness the termites cannot make up the loss by even a long sojourn in a highly saturated atmosphere. This view is further substantiated by the data presented in Experiment 4, below, from which it is evident that rapid drying extracts the major portion of the water in about eight hours. In Group III the desiccation of the animals probably reached at the end of the first 8 to 10 hours the same degree as that attained by Group I at the end of four days. But with Group III the remaining forty odd hours of dryness did not serve to make any material additions to the water loss. Experiment 4 indicates that at this stage of drying water becomes very difficult to remove and it also must be remembered that the animals of Group III were consuming considerable amounts of cellulose which, although dry, nevertheless must have contained a slight quantity of hygroscopic water.

TABLE III

The Water Loss and Viability of Termites under Varying Degrees of Atmospheric and Food Moisture

	Group I Moist air 22 hours per day. Dry air and food 2 hours	Group II Moist air 16 hours per day. Dry air and food 8 hours	Group III Moist air 12 hours per day. Dry air and food 12 hours
Average initial weight in mg.	33.4	37.3	33.7
Average weight after 4 days in mg.	26.0	29.6	25.9
Water loss in per cent	22.4	20.8	23.1
Per cent mortality after days specified: 4	8	4	0
6	48	4	4
8	80	44	24
12	96	96	76
15	100	100	92

From these findings the conclusion seems to be justified that the termite cannot utilize the water in the atmosphere to *replace* that lost by evaporation. The viability data indicate that during prolonged exposures death occurs primarily as a result of drying, and secondarily, from the starvation which accompanies it. The course of mortality was similar in all three groups but life was most prolonged in the twelve-hour group, and least prolonged in the two-hour group. Since the former had a longer feeding period, it seems possible that they received slightly more nourishment despite the general unavailability of the dried wood. Nevertheless, the fact that practically none survived more than fifteen days is additional evidence that dry food, even when accompanied by long periods of atmospheric saturation, will not support these animals, and that the moisture in the atmosphere can function only to prevent evaporation and thus to *diminish the water loss*. If now we exclude the water vapor surrounding the termite as a source of water we are forced to conclude that normally the water is taken in with the food.[2]

Experiment 4. The uniformity in the water loss of the termites in Experiment 3, even with a 600 per cent variation in time of exposure to very dry air, raises the question of just how rapidly the loss of water occurs, for apparently the first 20 per cent of the water is lost within 8 hours. To make certain of this point 25 termites were placed in a desiccator over anhydrous calcium chloride and were weighed at frequent intervals. No food was given. From Table IV it may be seen that the total water loss rose as high as 28 per cent after 18 hours exposure, but that the termites were rapidly dying. Since there was no food, it was impossible for the termite to get water, even in traces from wood, but the sharp fall in water content to a 20 per cent loss in 8 hours fits in with the result obtained in the previous experiment. In fact, here the death rate was even more rapid than in Experiments

TABLE IV

Rate of Water Loss in Termites under Dry Conditions

Hours in desiccator	Average weight per termite	Water loss	Rate of water loss
	mg.	*per cent*	*mg. per hour per termite*
0	51.0	0	
4	45.3	11.2	1.4
8	39.9	21.6	1.3
18	36.7	28.4 (Eight dead)	0.35

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[2] Concerning the form in which the water may be ingested it may be pointed out that in addition to "free" water in the intercellular spaces of wood the hygroscopic water may be of importance. In short leaf pine at a relative humidity of 100 per cent (but with no condensation) the amount of water held in this way is approximately 30 per cent (Schorger, 1926). All forms of cellulose may absorb hygroscopic water up to a concentration of 17 per cent. The ability of the termite to exist in relatively dry places (but not completely dry) may depend upon the availability of this water after digestion of the cellulose in the gut of the termite.

2 and 3 (18 to 20 hours compared with 10 to 15 days), a difference which again may be correlated with the presence or absence of small amounts of water contained in wood. Our observations indicate that the termite survives a water shortage quite well until the loss reaches about 20 per cent of the normal fresh weight. At this point the animals begin to become moribund. Death is certain when the loss reaches about 28 per cent.

In conclusion, the chief results of this study may be briefly summarized. *Termopsis* loses water rapidly when placed in dry air, and unless the loss is compensated it may be fatal. The water for replacement is provided as liquid in the food. The animal is unable to take up water vapor actively from even a moisture-saturated atmosphere.

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